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Investigation and evaluation of a system for continuing regeneration of adenosine nucleotides.

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INVESTIGATION AND EVALUATION OF A SYSTEM FOR CONTINUOUS REGENERATION OF ADENOSINE NUCLEOTIDES

submitted by

JOHN HUBBLE

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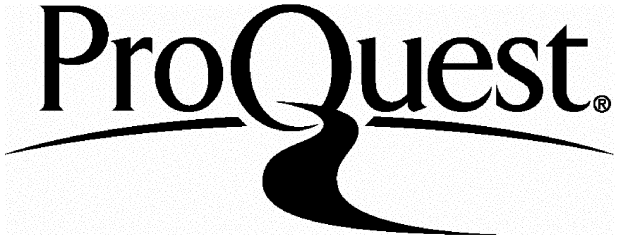

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SUMMARY

Three problems concerning the operation of enzyme reactors for the recycle of ATP were investigated.

The potential of a simple enzyme thermistor system for monitoring reactor effluent was studied. Initial results from physical characterisations showed the interdependence of operating parameters on overall detector sensitivity. Subsequent results demonstrated the detection of urea using an immobilized urease column. Attempts to improve the flexibility of the system by replacing covalently bound enzymes with an adsorption system proved unsuccessful.

Ultrafiltration reactors were considered the most suitable type for cofactor requiring systems. The effects of various operating parameters on the problem of concentration polarisation were studied. Results indicated that transmembrane pressure drop was not linearly related to protein concentration at the membrane, as determined by the 'Gel Polarisation' model. The degree of concentration polarisation was found to be a function of temperature, tangential flow and ionic strength of the solvent. The possibility of in situ removal of denatured enzymes was considered. It was found that with suitable adjustments to the operating conditions, proteases could be profitably used for this purpose.

A simple two step coupling procedure was developed for binding ATP/ADP to dextran. In the preliminary step, dextran was activated using a bis oxirane in a reaction time of 45 minutes. Subsequent binding

of ATP to activated dextran was found to be time dependent allowing a range of complex loading to be produced. The resultant complex was shown to be active with a range of enzymes. Essentially all cofactor groups were shown to be available to hexokinase at the highest loading produced. The effect of cofactor loading on the apparent kinetic constants obtained with hexokinase suggests that the cofactor groups are all equally available. The potential of this complex was demonstrated in a small scale ultrafiltration reactor with hexokinase and acetate kinase.

CONTENTS

INTRODUCTION

BACKGROUND

Continued advances in the fields of biochemistry and chemical engineering have led to areas of overlap between the two disciplines. For the sake of convenience these have been linked under the global heading of biotechnology, and more specifically biochemical and genetic engineering (Spinks et al., 1980).

These titles allied with a great deal of current interest gives the misleading impression of a new area of research. In fact, Man's use of biochemical systems can be traced to prehistoric times. Obvious examples which can be cited include the brewing of alcoholic beverages, the fermenting of soured milk to form cheese, and the use of yeast in the baking of bread (Haas, 1976).

The recent surge of interest in this area could be said to be the result of a greater understanding of the processes involved, leading to a greater flexibility in approach. Atkinson, (1974) considers a simplified scheme of historical development :-

Pre 1800 - a period of ignorance.

1800-1900 - a period of discovery.

Post 1900 - a period of industrial development.

Fermentations

Developments in the field of microbiology, starting with the work of Pasteur, leading to the concept of aseptic techniques and specific growth media, have widened the range of organisms and hence reactions available (Aiba et al., 1973). The main classes of fermentation products are antibiotics, steroids, enzymes,alcohols and organic acids (Aiba et al., 1973). The intrinsic nature of fermentations limits their usefulness for catalysis of specific reactions, therefore, methods of eliminating unwanted side reactions are required.

Immobilized Cells and Organelles

Immobilized cells offer advantages of stability, low nutrient requirement, and greater ease of control over traditional fermentations (Cheetham, 1980). In addition many metabolic pathways remain intact, allowing complex reactions to be carried out. Problems of cofactor regeneration are avoided allowing degradative and synthetic reactions to be linked (Jack and Zajic, 1977. Cheetham, 1980).

Methods of immobilization fall into the same catagories as those used to immobilize enzymes and a wide range of methods have been investigated (Klein and Wagner, 1979). Several industrial processes have been based on immobilized cell systems; notably the production of L-aspartic acid from ammonium fumarate (Chibata et al., 1974. Tosa et al., 1974) and the conversion of fumaric to malic acid (Chibata et al., 1975. Yamamoto et al., 1976, 1977).

Major disadvantages include side reactions and contamination from cell lysis. In some cases, pretreatment can reduce side reactions (Sato et al., 1976. Yamamoto, 1974), however, this still remains a major problem.

To further reduce unwanted side effects some workers have investigated the possibility of immobilizing cell fragments or organelles (Pace et al., 1976). A recent example of this work is the immobilization of chloroplasts in an attempt to produce photolytic biocatalysts (Gisby and Hall, 1981).

Discrete Enzyme Systems

An important advantage of enzyme systems is their high specificity, in some cases allowing discrimination between two optical isomers of the same compound (Ogston, 1948). In many cases a high level of catalytic activity is shown. Mahler and Cordes (1971) quote a range of turnover numbers between 10^2-10^6 molecules/minute. These high catalytic rates have the additional advantage of being obtainable under mild conditions of temperature and pH (Vieth and Venkatasubramanian, 1973).

The use of purified or partially purified enzymes allows higher catalyst densities to be used. Schnyder (1974) demonstrated advantages of using high activity glucose isomerase, eliminating unwanted side reactions during the production of fructose. For many enzymes the cost of production precludes use in batch systems. As most native enzymes are soluble, methods of delimiting them within a reactor must

be considered. Zaborsky (1973) defines this immobilization as "the physical confinement or localisation of enzymes during a continuos catalytic process".

Large Scale Preparation of Enzymes

Commercial microbial enzymes date back to 1890, with the production of a mixture of amylolytic and proteolytic enzymes extracted from Aspergillus oryzae (Aunstrup, 1979). In 1960 Beckhorn stated that although enzymes from plants and animals were more important, microbial enzymes would, for both economic and technical reasons, play an increasingly important role. This prediction was subsequently born out. In the early 1970's the bulk of commercially available enzymes were extracellular, from prokaryotic organisms (Lampen, 1972. Solomons, 1977).

The restricted types of extracellular enzymes necessitates investigation of extraction procedures for intracellular enzymes. These methods have been considered by Dunnill and Lilly (1972) with respect to continuous processes. When considering the production of enzymes from microbial sources, it is important that the chosen organism can be safely handled on a large scale (Dunnill and Lilly, 1972). In some cases, the organisms used must be acceptable to regulatory authorities.

Reduction of processing costs can be acheived by enhancing intracellular levels of required enzymes. Methods include induction (Takasaki, 1971), strain selection (Lee et al., 1972) and mutation (Diers, 1976). In

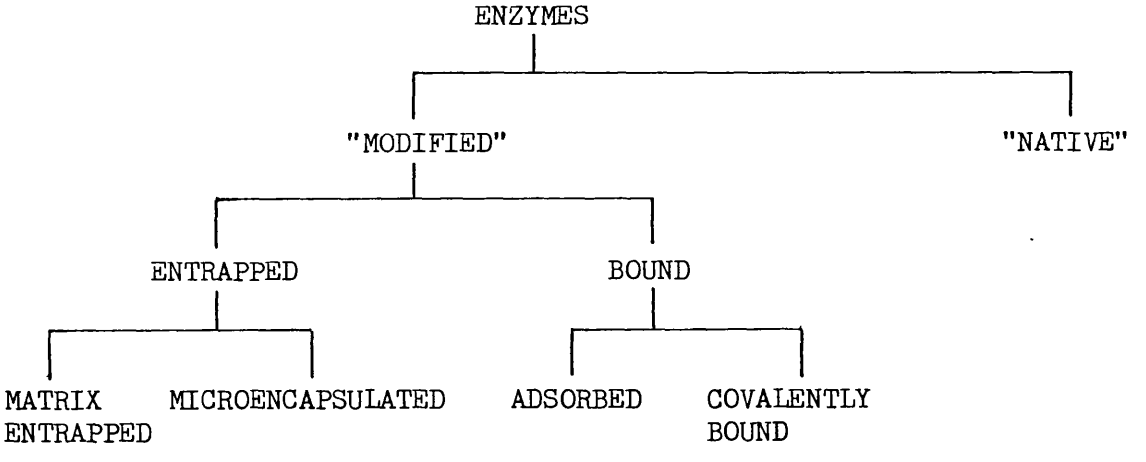
certain cases dramatic improvements can be obtained. Norvich and Horuichi (1961) report a mutant of E. coli producing 20% of intracellular protein as β-galactosidase.

Choice of fermentation conditions is critical in many cases (Lilly, 1979). Factors reported to affect growth rate and enzyme yield include, carbon source concentration (Sargeant, 1974), dissolved oxygen tension (Harrison, 1972. Wimpenny, 1969) and, in continuous systems, the dilution rate (Dean, 1972). The effect of fermentation conditions on the subsequent harvesting and extraction of the enzymes must be considered. Time consuming operations, such as centrifugation and filtration may well result in loss of enzyme activity (Thurston, 1972). The mechanical ease of disruption should also be considered (Wang et al., 1979), in some cases this has been shown to be affected by the fermentation media (Gray et al., 1972). Recent approaches to industrial scale purification include development of aqueous two-phase systems (Kula, 1979) and affinity chromotography. The important techniques have been reviewed by Melling and Phillips (1975).

Recent developments in genetic engineering have generated a great deal of interest. This has been fuelled by the announcement that I.C.I. had successfully increased the metabolic efficiency of the microorganism Methylophilus methylotrophus, used in their single cell protein plant. The improvement of approximately 4% was acheived using genetic engineering techniques (King, 1982). Another important development is the production and isolation of monoclonal antibodies, which should aid the transition of affinity chromotography from laboratory to industrial technique.

IMMOBILIZED ENZYMES

Developments in enzyme immobilization techniques during the late 1960's and early 1970's required that efforts be made to rationalise the associated terminology. In 1971 an ad hoc committee discussed the possible standardisation of terms used in the field of enzyme technology. Four resultant recommendations were made concerning methods of immobilization, expression of kinetic parameters, stabilisation criteria and covalent coupling data (Sundaram et al, 1972). These workers propose the following classification of immobilized enzyme systems.



Further developments have led to new methods which can be considered as techniques in their own right. Ultrafiltration, and the use of multifunctional reagents to form crosslinked enzyme complexes, have been considered separately in review articles (Zaborsky, 1973).

Covalent Binding

Covalent attachments of soluble enzymes to an insoluble support is the

most common technique for the immobiliztion of enzymes (Zaborsky, 1973). The earliest example of a synthetically produced enzyme-support complex was described by Grubhofer and Scheith (1954) who immobilized several enzymes on diazotized polystyrene.

Subsequent work has shown the feasibility of immobilization using a wide range of supports. This area has been extensively reviewed (Zaborsky, 1973. Schlunsen et al., 1979. Messing, 1978). Factors considered include protection of the enzyme during immobilization (Browne et al., 1968), functional groups available for attachment (Silman and Katchalski, 1966) and chemical characteristics of carriers (Manecke et al., 1979). Palm (1979) considers stabilization as a major aim of immobilization. Many workers have shown increased stability of certain immobilized enzymes over their soluble counterparts (Weetall, 1970. Chibata and Toza, 1976). However, Johnson (1979) demonstrates exceptions to this rule and points out the dangers of generalisations based on limited data.

Adsorption

Adsorption of enzymes onto an insoluble carrier is the earliest technique used for immobilization (Nelson and Griffin, 1916); (as the process stems from physical rather than chemical interactions the cost of linking reagents is avoided). The flexibility of the interactions suggest that distortion of the enzyme is the less likely to occur than in covalently bound enzymes. Katalski et al. (1971) and Brockman et al. (1973) consider this to be the closest in vitro system to a membrane bound enzyme.

The nature of the interactions is such that adsorption is a reversible process. It has been observed that changes in process conditions can cause desorption (Mitz et al., 1956, 1961). These changes include pH (Messing, 1970), ionic strength (Chung et al., 1968) or the presence of substrate (Goldman and Lenhoff, 1971).

Despite this disadvantage, adsorption has been frequently used in industrial processes. Examples include, glucose isomerase adsorbed onto DEAE cellulose (Schnyder and Logan, 1974), and amino acylase on DEAE sephadex (Chibata et al., 1972). Adsorption has been demonstrated on a range of supports including carbon (Nelson and Griffin, 1916), alumina (Nelson and Hitchcock, 1921), cellulose (Mitz and Schulter, 1959) and silica (Vorobeva and Poltorak, 1966). Methods of adsorption have been reviewed by Zaborsky (1973) and Johnson (1979).

Entrapment

This technique relies on physically restricting the movement of the enzyme by forming a polymeric matrix about the enzyme molecules. The large molecular weight of the enzyme prevents loss due to elution but small molecular weight reactants and products are freely diffusible through the network.

Early reports of lattice entrapments include the immobilization of urease and catalase in silica gel (Dickey, 1955). However, with highly charged polymers, Zaborsky (1973) suggests a proportion of the observed immobilization to be accounted for by adsorption.

Early workers favoured the use of polyacrylamide matrices. These have the advantage that pore size and rigidity can be controlled by varying the concentration of the two monomers (acrylamide and N,N',-methylene bis acyylamide). The effects of these concentrations on the activity of lactate dehydrogenase was studied by Hicks and Updike (1966). A range of catalytic methods have been used for initiating polymerization. In principle, the most attractive method is the use of X-rays (Dobo, 1970) as this allows the best control of reaction rate allowing minimization of the resultant heat build up.

Other polymers used include, silastic resins (Pennington et al., 1968) and starch gels. A recent development is the entrapment of small droplets of an aqueous solution of one or more enzymes into a fibrous matrix (Dinelli et al., 1975). Cellulose triacetate became the polymer of choice, factors affecting this decision being, cost, mechanical properties and good resistance to both chemical and microbial attack (Marconi and Morisi, 1979). For a review of industrial applications of fibre entrapped enzymes see Marconi and Morisi (1979). Disadvantages of entrapment include, restriction on the molecular weight of the substrate (Johnson and Whately, 1971). In some cases leakage has been reported to be a problem (Bernfield and Wan, 1963. Guilbault and Das, 1970), however, Dinelli et al. (1975) claim that control of pore size can minimise this problem in the case of spun fibres. In certain situations pore size restrictions can be an advantage as the matrix can protect the enzyme(s) from antibodies in vivo and also prevent microbial attack (Bernfield and Wan, 1963).

Microencapsulation

The formation of microcapsules with semipermeable membranes for entrapping enzymes was first reported by Chang (1964). Two general methods have been used for the preparation of microcapsules. The two methods stem from entirely different underlying principles. Coacervation, a physical phenomenon, is the separation of phases in a polymer solution. Enzymes in aqueous solution are emulsified with an organic solvent containing polymer. Microcapsule formation results from the lower solublity of the polymer at the interface of the microdroplet. This method has been used to produce microcapsules from a range of compounds including Collodion (Morgensen and Vieth, 1973), polystyrene (Chang, 1964) and ethyl cellulose (Kitajima et al., 1969).

The alternative method, interfacial polymerisation, results from the formation of polymer at the interface of two immiscible solvents. One reactant is soluble in both organic and aqueous phase, the other in solely the aqueous. In this case, the microcapsule is formed by chemical reaction. The most common polymer (Nylon) is usually formed from 1,6-hexanediamine and 1,10-decanoyl chloride, (Chang and Poznansky, 1968). Other polymers include crosslinked proteins (Chang, 1964), polyamines (Shiba et al., 1970) and polyurea (Mori et al., 1973).

Major disadvantages are found in the high protein concentration required in the aqueous phase (Bogulaski and Janik, 1971) and in common with lattice entrapment, the restrction to small substrate molecules (Chang, 1964).

The problem of protein concentration has been alleviated by the use of inert protein to increase concentration (Sundaram, 1973).

In a review in 1975, Chang examined the potential therapeutic uses of microcapsules. The polymer gives protection from antibodies, and microcapsules compare favourably with cells for mechanical properties.

Ultrafiltration

An alternative to the use of immobilized enzymes is to delimit native enzymes in a membrane reactor. Suitable ultrafiltration membranes retain soluble enzymes while small molecular weight reactants are free to pass through. One of the first suggestions for an ultrafiltration enzyme reactor came from Michaels in 1968, although earlier workers had used ultrafiltration techniques for protein purification and concentration (Blatt et al., 1965).

Van Oss (1970) classified semipermeable membranes into two general groups, isotropic - having a uniform structure throughout the entire wall, and anisotropic - having a smooth skin on one face. This asymmetry of the anisotropic membrane prevents macromolecules from entering and fouling the pores of the membrane.

Michaels (1968) proposed an alternative classification of ultrafiltration membranes, also with two general classes, microporous - having a highly voided structure containing interconnected small pores where flow is essentially viscous; and diffusive - which can be considered as a matrix where transfer occurs as a result of molecular diffusion. The diffusive membranes show poor solvent permeabilities

resulting from the thick semipermeable membrane. Limitations stem from difficulties in preparing thin, perfect polymeric membranes. However, it is possible to prepare anisotropic diffusive membranes. In this case a thin polymeric membrane is supported by an inert porous material.

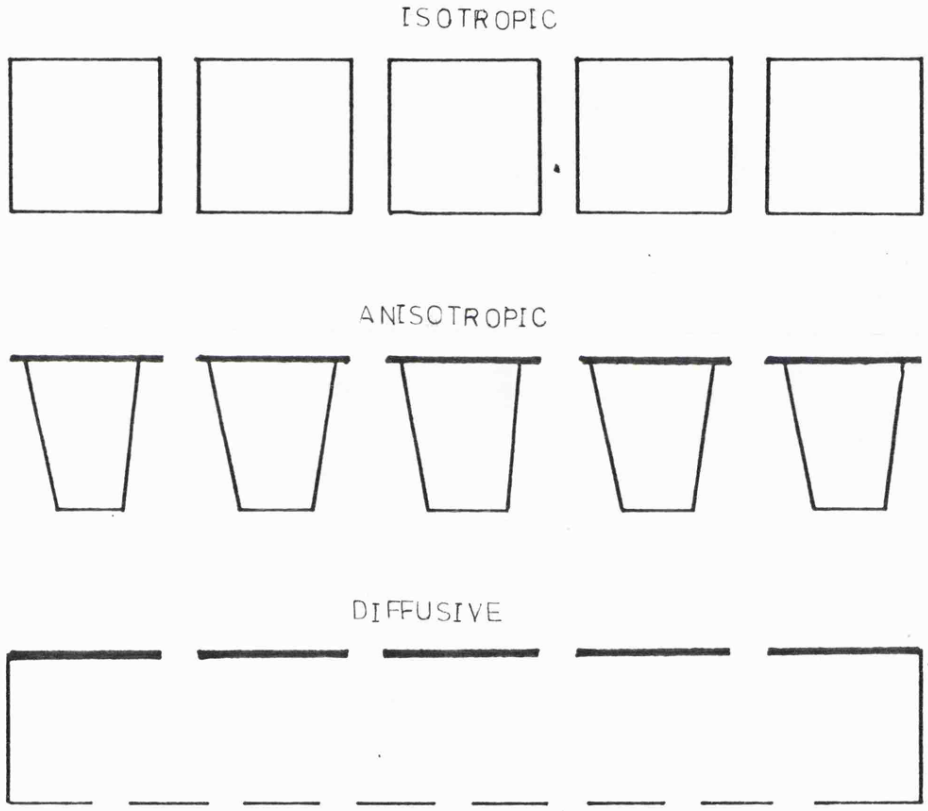


Fig. 1.1 TYPES OF MEMBRANE

Diagram (Charm and Lai, 1971)

The processes and limitations of ultrafiltration systems have been reviewed by several workers (deFilippi and Goldsmith, 1970. Wang et al., 1970. Porter, 1972).

Since the late 1960's several systems using soluble enzymes in ultrafiltration reactors have been published (Wykes et al., 1971. Butterworth et al., 1970. St^avenger, 1971). Usually there is no need for the enzyme(s) to be modified, however, in some cases modification may be advantageous. Examples include coupling enzymes to polymers to allow use of membranes with high molecular weight cut-offs, permitting an increased permeation flux (Wykes et al., 1971). In the case of proteolytic enzymes some protection from autodigestion can be acheived by modification (O'Neill et al., 1971).

Zaborsky (1973) considers the advantages of ultrafiltration to include simplicity of use, elimination of expensive linking reagents, higher catalytic activities shown by native enzymes and the ease in which several enzymes can be co-immobilized. This latter point has allowed the regeneration of coenzymically active, immobilized NAD in ultrafiltration cells (Mosbach and Mattiasonn, 1970. Wykes et al., 1975). In addition the kinetic properties of an enzyme in an ultrafiltration reactor should be predictable from its steady state kinetic properties, which can be easily determined in vitro.

The major disadvantage of ultrafiltration is the phenomenon of concentration polarisation, namely the buildup of solute molecules at the membrane surface. This build up of solute can severely restrict transmembrane flux and lead to apparent fouling of the membrane (Porter, 1972). In the case of proteins, if the concentration of solute at the membrane reaches the concentration of 15 to 20%, gelation can occur leading to the formation of a secondary membrane (Blatt et al., 1970. de Fillippi and Goldsmith, 1970). This solute deposition is countered by back diffusion into the bulk phase and eventually a

steady state is reached.

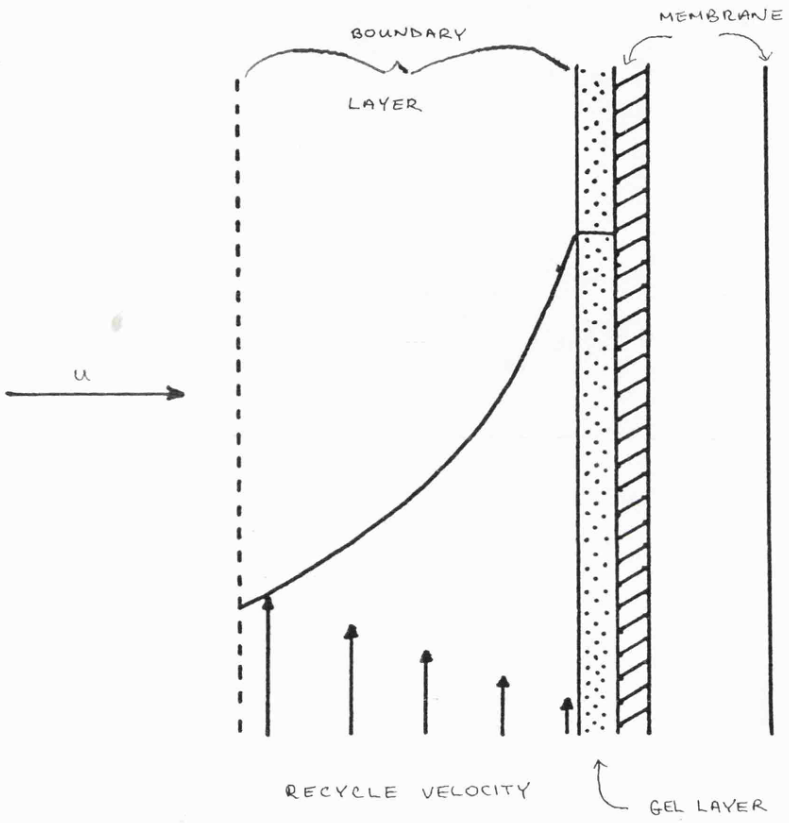


Fig. 1.2

Boundary Layer Formation in Ultrafiltration from Porter (1972)

This situation can be described mathematically by the following equation, based on the assumption that the solute is totally rejected by the membrane (Flaschel and Wandrey, 1979).

$$E_m = E_b \exp\left(\frac{u\,\delta_1}{D_e}\right)$$

where :- E_m is the enzyme concentration at the membrane

E_b is the bulk enzyme concentration

u is the linear velocity through the membrane

δ_1 is the thickness of the laminar boundary layer

D_e is the diffusion coeffecient for the enzyme

The exponential term can be regarded as the Boltzmann-factor, describing two opposing forces, in this case enforced flow and back diffusion.

An examination of the above equation shows that conditions can be adjusted such that a membrane reactor can be operated with the enzyme in a soluble form, well mixed throughout the reactor volume (homogenous catalysis). Alternatively, the enzyme can be polarised onto the membrane such that it operates in a packed bed situation (heterogenous catalysis). Methods utilising both approaches have been reported. Alfani et al.,(1979) and Greco et al., (1979) report the polarisation of several enzymes onto various membranes. Subsequent immobilization is achieved by addition of Human serum albumin such that gelation occurs. This technique was shown to result in dramatic increases in enzyme stability, however, such immobilized systems showed lower rates than corresponding soluble enzymes. This reduction

in rate was shown to be associated with substrate limitations arising from diffusion resistance within the gel layer (Alfani et al., 1979).

Despite possible advantages of stability associated with heterogenous operation, the majority of work has been published of homogeneous systems (Flaschel and Wandrey, 1979). Certainly if the use of soluble cofactors is required, homogeneous operation would appear to offer the most practical approach.

COFACTOR UTILISATION

Cofactors can be described as non-proteinaceous compounds, essential for certain enzyme reactions. Of approximately two thousand known enzymes about 40% require cofactors (Wang and King, 1979). The term cofactor covers a range of compounds from simple mono and divalent ions to highly complex organic molecules.

If industrial use is to be made of enzymes as catalysts the problem associated with provision of the neccessary cofactors must be investigated (Lowe, 1981). To simplify an assessment of this problem Wang and King (1979) classified the organic cofactors into three groups depending on their regeneration requirements. Inorganic ions (Mg^{2+}, Ca^{2+}, etc.) need not be considered in this scheme as they are cheap and act in a classical catalytic manner.

The first group covers those organic cofactors which are self regenerating; these are usually tightly bound to the enzyme through covalent or secondary bonds. These tightly bound compounds are often described as prosthetic groups (Mahler and Cordes, 1971) and include pyridoxal phosphate (vitamin B_6) and thiamine pyrophosphate.

The remaining compounds all need some form of catalytic regeneration. A distinction can be made between those which can be regenerated by catalytic oxidation with oxygen as the electron acceptor (including FAD, FMN and Lipoic acid) and those which require an alternative substrate for regeneration (including coenzyme A, NADH and ATP) (Baricos et al., 1976).

From an examination of their properties Wang and King (1979) suggest immobilization is required for freely diffusable organic, stochiometric cofactors. The most important of these from an industrial viewpoint are the five principle adenine nucleotide containing coenzymes, NAD, NADP, AMP, ADP and ATP (Lowe, 1981).

Synthesis of Cofactor Analogues

Wang and King (1979) consider two reasons for immobilizing cofactors. Firstly, to widen the scope of enzyme technology to include the use of oxidoreductases, ligases and other cofactor requiring enzymes. Secondly, to produce ligands for use in affinity chromotography. Lowe (1981) points out the importance in distinguishing between the ability of an enzyme to bind to an immobilized coenzyme and the ability of an enzyme to utilize a bound coenzyme in a catalytic reaction. Generally there are fewer constraints on the cofactor analogue in the case of affinity chromotography (Lowe and Dean, 1974). In cases where immobilized coenzymes are required to retain full activity, more stringent requirements have to be met for the reaction to be carried out at an economically viable rate.

To ensure that maximum activity is retained after immobilization, it is important to have some understanding of the interaction between coenzyme and enzyme. X-ray crystallographic studies on various dehydrogenases (Adams et al., 1973. Webb et al., 1973. Bränden et al., 1973. Buehner et al., 1973) have revealed a pattern of structure-function relationships. The workers suggest that the enzyme subunits are divided into two discrete functional domains, one binding coenzyme

the other substrate. The cofactor binding domains have been shown to display basic similarities in both structure and mode of coenzyme binding (Ohlsson et al., 1974). However, the substrate binding domains showed marked differences in structure as might be expected from the different specificities.

To date, all dehydrogenases investigated have shown marked similarities in secondary structure of coenzyme binding domains although the position of the domain relative to the primary structure may vary. The basic structure has been shown to comprise of six parallel strands of β-pleated sheet (βA-βF) and four helices (αC,αD,αE and αF) (Ohlsson et al., 1974) see Fig.1.3.

The binding is associated with the parallel β-pleated sheet, the cofactor being bound in an open conformation with the adenine and nicotinamide rings unstacked, about 14 Å apart.

In lactate dehydrogenase the hydrophilic adenine binding crevice has been well characterised (Adams et al., 1973), enabling the chemical interactions responsible for cofactor orientation to be determined. Studies with inhibitors have shown that other aromatic molecules are bound; this phenomenon has been demonstrated with a range of compounds, salicylate (Grisolia et al., 1970), 1-anilino-8-napthalene sulphonate (Lu and Anderson, 1973) and Cibacron Blue F3G-A (Lowe, 1979 (a)), being a few cited examples. The relationship between enzyme and cofactor in the lactate dehydrogenase-NAD^+-pyruvate abortive complex, as proposed by Adams et al., (1973), can be seen in Fig.1.4.

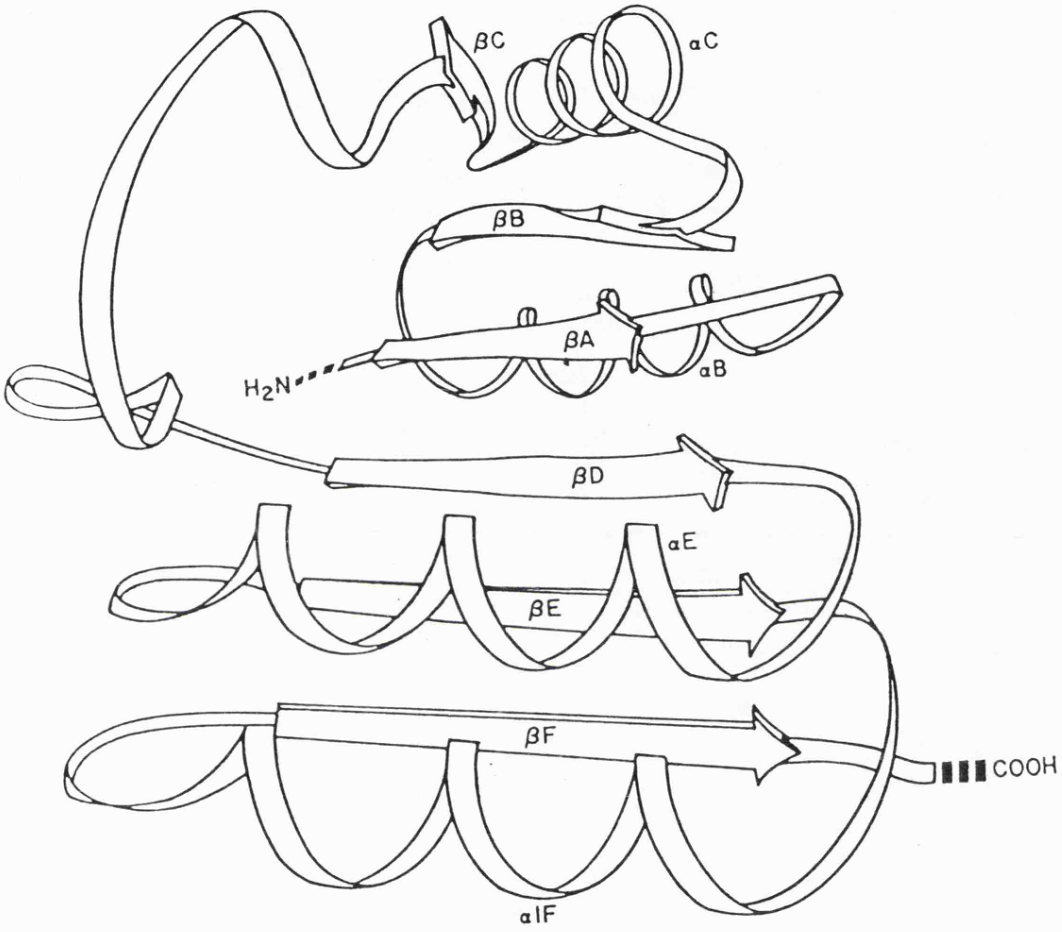


Fig. 1.3 Structure of Dehydrogenase Cofactor Binding Site

(taken from Ohlsson et al., 1974)

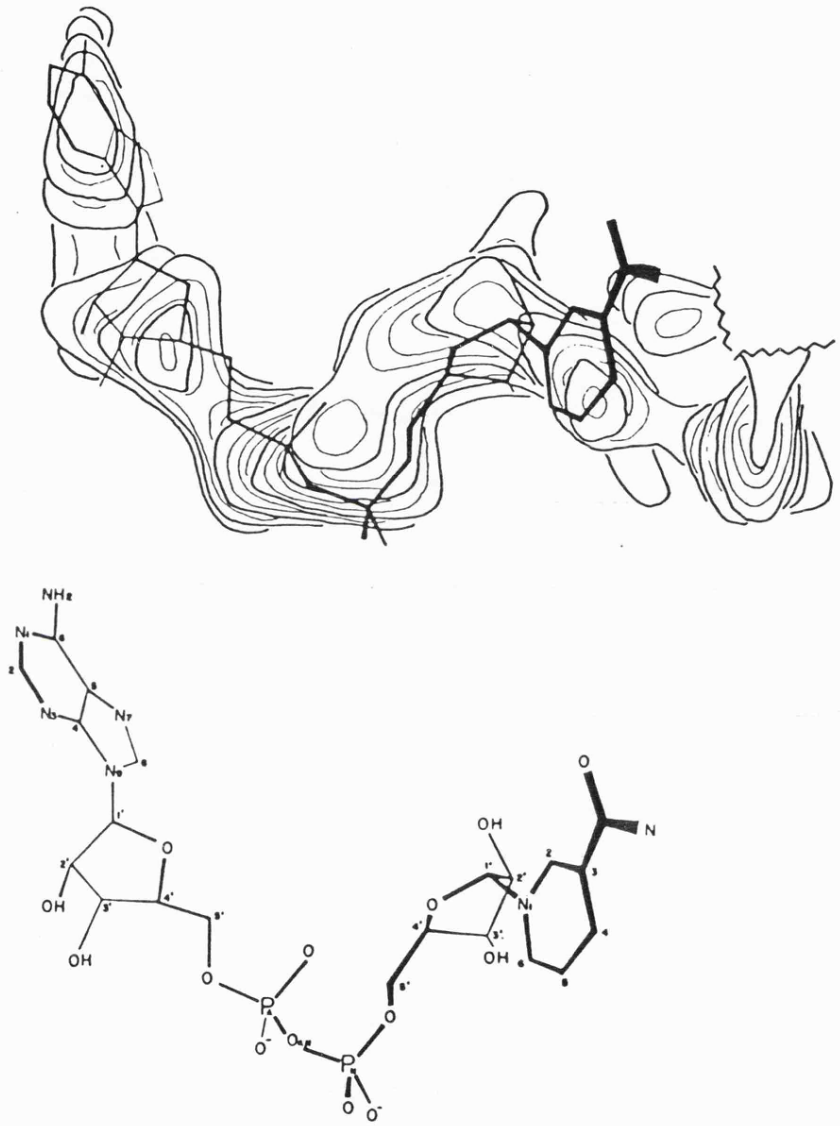


Fig. 1.4 Relationship between Lactate Dehydrogenase and NAD^+

(taken from Adams et al., 1973)

Work on a wider range of coenzyme requiring enzymes has shown a striking structural similarity throughout. Phosphoglycerate kinase, comprising a single polypeptide chain, shows essentially the same ADP binding domain as the dinucleotide linking fragment of lactate dehydrogenase (Blake and Evans, 1974). A similar result has been demonstrated with adenylate kinase (Schultz and Schirmer, 1974) and monomers of yeast hexokinase (Steitz et al., 1973). These results, suggesting a common primordial mononucleotide binding protein, have led to the proposal of a mutual evolutionary history (Blake and Evans, 1974).

This understanding of the structural aspects of cofactor binding would appear applicable in most cases and provides a useful guideline for developing analogues. However, Lowe (1981) concludes that variations between individual enzymes cannot be discounted; and potential substitution positions should not be disregarded solely on a basis of structural considerations.

The most commonly used position for modification is the N^6amino group of the purine base. From the structural considerations previously discussed, this is an ideal site, as this group protudes from the binding pocket. The first coenzyme analogue developed for immobilization, to be chemically defined was N^6-(6-amino-hexyl)-AMP. These analogues are usually synthesised by nucleophilic displacement; various leaving groups have been used including 6-chloro (Guildford et al., 1972), 6-mercapto (Craven et al., 1974 (a)) and 6-methyl sulphate (Eckstein et al., 1975). The conditions required for these reactions affect their general suitability. The method proposed by Eckstein

et al., (1975) allows substitution with 1,6-diaminohexane to be carried out at room temperature over a period of approximately 20 minutes. This avoids the disadvantage of high temperatures (80-95°C) and alkaline conditions associated with the other methods cited.

Other reactions considered include reaction of unmodified NAD with succinic anhydride to form a hemisuccinamide (Wykes et al., 1972), carbamylation with hexamethylene dicyanate to generate 6-amino purine derivatives (Yamakazi et al., 1977), and reactions with formaldehyde to give N^6-hydroxymethyl derivatives (Yamakazi and Suzuki, 1978).

Many of these reactions are suitable for synthesis of 'half' analogues only, in view of the harsh conditions required. A potential solution to this problem is the derivatisation of AMP, followed by enzymic condensation with nicotinamide mononucleotide (Craven et al., 1974 (b). Harvey et al., 1974 (a)).

A procedure commonly used for formation of N^6-substituted derivatives, involves quarternisation of the N^1 position of the adenine ring by alkylation followed by an alkaline Dimroth rearrangement to give the N^6-substituted derivative (Wilson and McClosky, 1973). In view of the limited stability of the oxidised form of NAD in the alkaline media required for the Dimroth rearrangement it is usually reduced to the more stable form by enzymic (Lowe and Mosbach, 1974) or chemical (Lindberg et al., 1973) methods. The final derivatives are subsequently oxidised enzymically to allow acid degradation of all unwanted products, and purification by ion exchange chromotography. This method has also been used to produce N^6 substituted analogues

of Coenzyme A (Rieke et al., 1979). More generally the method can be considered for all adenine containing cofactors (Lowe, 1981).

The Dimroth rearrangement method has been used in conjunction with a number of alkylating agents including iodoacetic acid (Mosbach et al., 1976), 3,4-epoxybutanoic acid (Zappelli et al., 1975), 3-propriolactone (Muramatsu et al., 1977) and aziridine (Weibel et al., 1974). In cases where the resultant analogues have only short spacer molecules (iodoacetic acid, 3,4-epoxybutanoic acid, 3-propriolactone and aziridine) it is usual to extend the molecules by attachment of supplementary spacer molecules (Lowe, 1981).

The alternative point of attachment to the purine base is the C_8 carbon atom. This position is susceptible to direct electrophilic substitution, direct bromination of the nucleotide allows subsequent nucleophilic displacement by a suitable nucleophile. A typical example is 1,6-diaminohexane which has been used to form the 8-(6-aminohexyl)-amino derivatives of AMP (Jergil et al., 1974), ADP and ATP (Lee and Kaplan, 1975, Lee et al., 1977) NAD and NADP (Lee et al., 1974. Lee and Kaplan, 1975). Other methods include nucleophilic displacement using thiol containing reagents. Chan and Hassid (1975) used cysteamine to generate 8-(2-aminoethyl)-thio-ATP, while Zapelli et al. (1976) substituted 8-Br-NAD^+ with 3-mercaptopropionic acid to yield 8-(2-carboxethyl)-thio-NAD^+.

Other positions used for substitution are the vicinal OH groups of the ribose moiety, the terminal phosphates of coenzymes such as ADP and

ATP, and also linkage to the pyrimidine moiety of NAD^+. Linkage through the ribose hydroxyl groups involves oxidations to aldehyde groups with periodate; subsequent reaction with a dihydrazide allows coupling to CNBr activated agarose (Lamed et al., 1973. Wilchek and Lamed, 1974). Lamed et al. (1973) report synthesis of AMP, ADP, ATP, NAD and NADP derivatives produced in this manner, however, the extensive modification of the ribose moiety restricts their use to a limited number of enzymes.

Immobilization through the terminal phosphate group has been achieved using several techniques. One convenient and widely used method involves the phosphorylation of 6-amino-hexane-1-ol. This is subsequently reacted with the required nucleotide to form an assymetric pyrophosphate (Barker et al., 1974). This method has been used to immobilize both AMP and ADP (Harvey et al., 1974 (b). Barker et al., 1974). An obvious disadvantage of position of attachment is the unsuitability of the resulting analogue for use in reaction catalysed by kinases, i.e. phosphate exchange. Relatively little work has been published on immobilization to the pyridine moiety of NAD; however, in cases where adenine modifications affect binding this approach can be considered.

Support Matrices

Although the majority of work published concentrates on the attachment of coenzymes and other ligands to insoluble matrices for affinity chromotography purposes, there are reports of soluble cofactor complexes based on a range of different supports.

The most popular soluble support to date would appear to be soluble dextran. Methods have been published for the coupling of a range of coenzymes, including Coenzyme A, (Rieke et al., 1979), NAD, (Weibel et al., 1974), ADP and ATP, (Gacesa and Whish, 1978), (Yamazaki and Suzuki, 1978), to this matrix. All but one of the above methods uses the cyanogen bromide activation method (Axen et al., 1967). As this linkage has been shown to be unstable under certain conditions, leaching of cofactor can occur. This is not significant with compounds having multipoint attachment, e.g. proteins, however, in the case of cofactors, severe use limitations can be imposed.

The method of Rieke et al. (1979), involves reaction of the amino group of a derivatised cofactor with epoxide activated dextran, thus forming a stable alkyl linkage.

An approach that is claiming increasing interest is the use of acrylic polymers (Fuller et al., 1980. Le Goffic et al., 1980 and Fuller and Bright, 1977). The advantages of this technique are the number and variety of acrylic monomers available for polymer synthesis, allowing a great deal of control over the resulting polymer (Fuller and Bright, 1977). The disadvantage of this early preparation was its low enzymic activity (5%), however, subsequent reports have been more promising. Le Goffic et al., (1980) quote a rate of derivatised NAD reduction of 35-81% of the native form. Fuller and Bright (1977), found an activity of 30% with NADH bound to a copolymer of methacryl choline and an epoxide containing monomer.

Zappelli et al., (1978) report the binding of NADP to polyethyleneimide.

They found that N^6-(2-hydroxy-3-carboxypropyl)-$NADP^+$ coupled to polyethyleneimide formed a stable complex. After strorage for six months in concentrated aqueous solution at 4^oC, no loss of bound nucleotide was observed, however, the enzyme reducible cofactor levels dropped to 80-85%.

An interesting alternative to the above supports is proposed by Coughlin et al., (1976). The technique involves the covalent coupling of NAD to alginic acid using a diepoxide. The resulting complex, showing cofactor activity with alcohol dehydrogenase, can be precipitated or solubilised by adjusting the pH of its surroundings. This phenomenon simplifies the ease of separating complex from unbound reagents and has obvious advantages if the complex needs to be recovered from a reactor.

At present there would appear to be no clear cut favourite amongst the supports used. The disadvantages of the cyanogen bromide activation methods and the complex methods of some cofactor derivatisation limit their potential for use in large scale continuous reactors. The use of diepoxides as described by Sundberg and Porath (1974) offer a simple, convenient method of forming a stable complex with a range of support materials. This allows choice of support to suit the reactor conditions required.

Cofactor Regeneration Systems

Having considered the derivatisation and immobilization of cofactors for use in enzyme reactors the problem of in situ regeneration must

be investigated. Discussion will be restricted to systems requiring nucleotide cofactors.

Regeneration techniques fall into three classes, electrolytic, chemical and biochemical. The most widely investigated reaction being the oxidation/reduction of NAD(P).

Recycle of NAD(P)

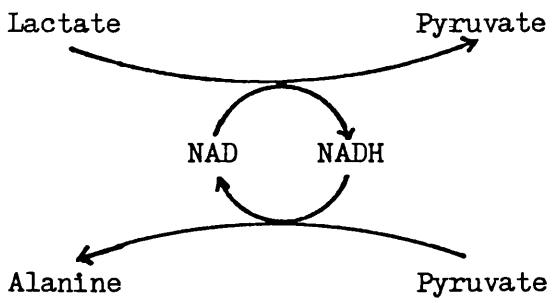
Chemical regeneration systems have received less attention than those utilising enzymes; however, many such systems have advantages of stability and cost. The majority of published work concerns the use of chemical redox couples for the regeneration of NAD(H) including sodium dithionite with horse liver alcohol dehydrogenase (Jones et al., 1972), methylene blue/H_2O_2 with glycerol dehydrogenase (Wagner et al., 1964) and sepharose bound actiflavin/H_2O_2 with lactate dehydrogenase (Mansson et al. 1976). Possible problems arise from difficulties in separating these small molecular weight catalysts from the reaction products. Jones et al., (1976) consider the major disadvantage to be low efficiency of chemical compared with other systems.

Alternatively, enzymically produced NADH can be regenerated electrochemically (Coughlin et al., 1975. Elving, 1976. Aizawa et al., 1975, 1976. Wallace and Coughlin, 1977). A specific example is the oxidation of NADH using an electrolytic method without the use of electron mediators (Aizawa et al., 1975). The oxidation appears to proceed in one step by a two electron reaction. The electrolysis was carried out for 100 minutes with an anode potential of 0.7 versus the

saturated calomel electrode. This gave a yield of 92% NAD which was shown to be active with ethanol and yeast alcohol dehydrogenase. The slow rate of this reaction casts doubt on its suitability for reactor applications; however, this technique may prove valuable in the design of enzyme probes for chemical and biochemical analysis (Blaedel and Jenkins, 1976).

The most widely investigated methods of regeneration are those utilising a second enzyme for the return reaction. These methods have the same advantages over chemical methods as have previously been discussed, namely specificity, mild conditions and high catalytic rates. A wide range of reaction schemes have been studied; however, the majority require a cosubstrate not involved in the primary reaction. Lowe (1981) has tabulated the components of some common regeneration systems.

Of greater interest however, are those schemes where the product of the first reaction is the substrate of the second. Such schemes avoid the additional cost of a second substrate and reduce problems of downstream processing by reducing the number of components in the product outlet. An example of this is the method proposed by Davies and Mosbach (1974) :-



An alternative method for cycling NAD(H) is the use of NADH oxidase from Streptococcus faecalis (Schmidt and Grenner, 1976). This system uses N^6-(2-aminoethyl)-NAD^+-dextran coupled with alcohol dehydrogenase and NADH oxidase. The number of cycles can be followed by the consumption of oxygen using an oxygen electrode. The velocity of the cycling reaction was 15% that with free NAD when using horse liver alcohol dehydrogenase and 8% when using yeast alcohol dehydrogenase. The advantage of this method is there is no contamination of the product stream as water is the side product of the second reaction. A similar principle has been applied to the regeneration of polyethyleneimine-FAD derivatives (Zappelli et al., 1978).

Recycle of AMP - ADP - ATP

The majority of work studying the regeneration of ATP has centred around enzymic methods. Langer et al. (1976) conclude that chemical methods are unsuitable on the grounds of expense and unfavourable conditions. Although considering the possible use of subcellular organelles e.g mitochondria and chloroplasts on the grounds of efficiency in the use of reactants, they are discounted as having too short a useful lifetime for practical processes. Enzymes are considered to offer the most promising approach both from considerations of specificity and stability. Langer et al. (1976) consider eight potential phosphotransferases in their assessments.

Chemical Methods

Several procedures have been proposed for the phosphorylation of adenosine and AMP (Cramer, 1964. Gutcho, 1970). One of the more

successful examples (Yoshikawa et al., 1968) is the phosphorylation of adenosine to AMP by reaction with phosphoryl chloride in trimethyl phosphate. The reaction requires a 200% molar excess and gives a yield of 88%.

The AMP formed can be phosphorylated to give ADP and ATP by reaction with dicyclohexylcarbodiimide and phosphoric acid in 50 to 10 fold molar excess respectively (Smith and Khorana, 1957). This method gives a 60% conversion to ATP with the remainder comprising ADP and higher phosphates. The higher phosphates formed are unsuitable for enzyme reaction, therefore, the efficiency of the method can be improved by reducing their formation. Moffat and Khorana (1960) found that they could be eliminated in favour of ADP by reacting the intermediate adenosine 5'-phosphoromorpholidate with bis-(tri-n-butyl-ammonium) pyrophosphate.

Although suitable for direct chemical synthesis of ATP these methods cannot be used for regeneration as the conditions required are incompatible with enzyme catalysis. In addition the methods require large excesses of expensive reagents; thus are not compatible with economic ATP regeneration, which has been shown to require a large ATP turnover (Baricos et al., 1975).

Cells and Organelles

Whole and disrupted cells have been used by a number of workers to synthesise ATP from adenosine and inorganic phosphate (Tanaka and Hironata, 1972. Tochikura et al., 1967). The specific reaction pathways involved are not completely understood but limited evidence

suggests that glycolysis is the primary route with excess glucose and P_i being taken up in the formation of glycolytic intermediates (Tochikura et al., 1967). The limited efficiency is the major disadvantage of the use of whole cells in this way.

A range of subcellular organelles have been studied with respect to ATP regeneration but all suffer from the drawback of limited stability (Langer, 1976).

A number of workers have investigated the use of mitochondria Lardy and Wellman, 1952. Arkles and Briniger, 1975). Typical half lives quoted for these systems are in the range 1-4 hours (Langer et al., 1976). Arkles and Briniger (1975) found that immobilization to glass beads did not lead to increased stability.

An alternative to mitochondria is the use of chloroplasts. These require illumination but neither oxygen or fuels, however, their operational life time is even shorter than mitochondria (Langer et al., 1976). In order to improve stability of the phosphorylating system attempts have been made to use subchloroplast particles. These particles have satisfactory storage stability when frozen but show a specific activity an order of magnitude lower than intact chloroplasts (McCarty, 1971).

Chromatophores, fragments of cytoplasmic membrane in the form of micelles, which can bring about essentially 100% conversion of ADP-ATP have been produced from photosynthetic bacteria (Pace et al., 1976). The storage half life has been improved 6 fold over native

chromatophores by immobilization in polyacrylamide (Yang et al., 1976). If the stability can be further improved, chromatophores may offer a viable system for ATP regeneration.

Enzyme Regeneration

While still showing the advantages of subcellular organelles, enzymes offer simpler, more robust systems which can be stabilised for extended periods (Whitesides, 1976). The phosphorylation of adenosine to AMP and of AMP to ADP is achieved using two enzymes;

Adenosine Kinase

Adenosine + ATP = AMP + ADP

Adenylate Kinase

AMP + ATP = 2ADP

These reactions will produce ADP at the expense of ATP, therefore, the final step, phosphorylation of ADP to ATP becomes crucial. This step offers a much wider choice of enzymes (Langer et al., 1976). These workers conclude that the most suitable reactions are those catalysed by the phosphotransferase enzymes, namely :-

ADP + X-P = ATP + X

At least eight examples exist where the $\Delta G^{o'}$ for hydrolysis of the phosphate donor is greater than ATP such that ATP production is favoured, Fig. 1.5.

Fig. 1.5

PHOSPHOTRANSFERASE	PHOSPHATE DONOR	BY-PRODUCT
Acetate Kinase	Acetyl Phosphate	Acetate
Arginine Kinase	L-phosphoarginine	L-arginine
Aspartate Kinase	4-phospho-L-aspartate	L-aspartate
Carbamate Kinase	Carbamyl Phosphate	Carbamate — NH_3 + CO_2
Creatine Kinase	Phosphocreatine	Creatine
3-phosphoglycerate Kinase	1,3-diphospho-D-glycerate	3-phospho-D-glycerate
Pyruvate Kinase	Phosphoenolpyruvate	Pyruvate
Ammonia Kinase	Phosphoramidate	NH_3

The preferred choice will depend on a range of factors including cost and stability of phosphate donors, cost and stability of the enzyme concerned, and comparability of reaction conditions with other reactions envisaged.

In their extensive review of ATP regeneration Langer et al., (1976) consider these factors and conclude that the most suitable candidates in the short term are acetate and carbamate kinase. The most severe limitation in most cases is the high cost of phosphate donors, 1-3-diphospho-D-glycerate being comparable to the price of ATP. Acetyl phosphate and carbamyl phosphate are both relatively cheap to produce (Whitesides et al., 1975. Metzenberg et al., 1960). This is also true of phosphoramidate, the phosphate donor in the ammonia kinase reaction, the disadvantage of this system being the low specific activity obtained and the marked instability of the enzyme (Marshall et al., 1972).

In a comparison of acetate and carbamate kinase systems, the most significant factor would appear to be the low stability of carbamyl phosphate in aqueous solution. Metzenberg et al., (1960) quote a half life of 2 hours at 30°C at neutral pH. This compares with 21 hours for acetyl phosphate under similar conditions (DiSabato and Jenks, 1961). This stability problem can be avoided in the case of carbamyl phosphate, by its synthesis in situ (Mokrash et al., 1960. Marshall, 1973). If the carbamyl phosphate is removed as rapidly as it is formed, the yield could approach 100%. There are two fundamental limitations to this approach. The rate of carbamyl phosphate synthesis is very slow compared with its enzyme catalysed removal, even at high phosphate and cyanate concentration. The

phosphate concentrations obtainable in the reactor are constrained by the enzymic requirements for Mg^{2+} ions, as the phosphorus salts of magnesium show low solubility products ($MgHPO_4$ 6.5×10^{-5}, $Mg_3(PO_4)_2$ 2×10^{-27})(Linke, 1965). These two factors leave acetate kinase as the most promising approach at the current time for systems using native cofactors and small scale.

When considering larger scale systems, the difficulties of by-product separation must be considered. From this standpoint, the carbamate kinase reaction has the advantage of producing carbamate which decomposes to give ammonia and CO_2, both easily removed. Acetate, the product of the acetate kinase reaction can also be volatilised by acidification to low pH. Other separation techniques have proved successful for removing acetate and other non volatile compounds from adenosine nucleotide, however, such methods increase the expense of the process (Gardener et al., 1974).

The conclusions of Langer et al. have been subsequently substantiated by the derivatisation of a large scale enzyme synthesis of ATP from adenosine and acetyl phosphate (Baughn et al., 1978). In a fed batch process of 240 hours duration, 60.9g of product was produced with an ATP content of 77%. This corresponded to a 36% yield based on adenosine and 15% based on acetyl phosphate. The enzymes used were entrapped in polyacrylamide beads and were recovered at the end of the process. The activities recovered were in excess of 90% for adenosine kinase and 70% for acetate kinase.

If derivatised cofactors are considered as substrates in a regeneration

reaction, the added factor of enzyme specificity must be considered. Gacesa (1977) found that ADP linked to dextran through the C_6 amino group showed no cofactor activity with carbamate kinase. He concluded that if this complex, suitable for inclusion in a membrane reaction, was to be developed, the specificity of other kinases would need to be investigated. This includes the primary as well as the recycle reaction.

ENZYME BASED SENSORS

Enzymes have been used for analysis for many years, a trend which is continuing with the increasing availability of highly purified enzymes. Bergmeyer in his extensive work "Methods in Enzymic Analysis" (1974) catalogues many such analyses.

Developments in immobilized enzyme technology have further increased the scope and flexibility of enzymic analyses. Bowers and Carr (1980) describe the advantages of immobilized enzymes as follows :-

1 Increased stability
2 Repetitive use of a single batch
3 The enzyme can be removed from the reaction mixture with no contamination
4 Long half lives with predictable decay rates
5 Easy preparation of unstable reagents

The use of immobilized enzymes in analysis can be split into two major categories. Firstly, the construction of a reactor which produces one or more products, subsequently detected by chemical probes or secondly, enzymes bound to transducers which detect physical or chemical changes in their immediate surroudings.

Analytical Reactors

Analytical reactors can be further divided into two groups depending on how the enzyme is immobilized. These come under the heading of

packed bed and open tubular reactors. The optimisation of both systems depends on three factors (Bowers and Carr, 1980), rate of conversion of substrate, dispersion of substrate causing reduced signals, and subsequent detection of product.

Open tubular reactors have received the most analytical interest resulting form their suitability in segmented continuous flow analysis. Traditionally these devices have been extensively used in analysis in clinical, environmental and pharmaceutical fields. This type of reactor first described by Skeggs (1957), is especially suited for reactions requiring long incubation times while maintaining precision. As each sample is in a disctrete liquid slug, axial dispersion is limited and potential sample overlap is avoided. The major restriction of this type of reactor is the surface area available for immobilizing enzymes. Comparisons between the surface area of a typical support used in packed bed reactors and that of nylon tubing suggest that columns would have to be a prohibitive length. However, Hornby and Cowards found reasonable conversion over relatively short lengths (3-10m) (Hornby et al., 1972. Campbell et al., 1975). This seemingly anomolous situation has been explained by work studying the effects of segmentation and coiling on the efficiency of the reactor. Engasser and Howarth (1972) concluded that for the same pressure drop, open tubular reactors provide higher mass transfer than packed bed reactors.

These approaches have been used in a wide range of determinations, the earliest being reported by Hicks and Updike (1966) for the detection of lactate and glucose using lactate dehydrogenase and

glucose oxidase followed by spectrophotometric detection of a coupled dye. Another example of interest is the detection of nitrate using nitrate reductase. This method published by Senn et al., (1976) had a postulated detection limit of 17 ppb. The advantages of this method led to its adoption by the U.S. Environmental Protection Agency as an official analysis technique.

The methods outlined above have been developed with analysis of discrete samples in mind. They are generally too complex to be used for continuous analysis of a product stream although a process could be monitored by taking samples at fixed time intervals.

Transducer Bound Enzymes

In this approach the enzyme(s) are immobilized in close proximity to a transducer which responds to change in its environment initiated by the enzyme reaction. The aim of this approach is to produce a cheap, robust sensor which is capable of giving a continuous response (Barker and Somers, 1978). Ideally the sensor would require no external reactants and be as non destructive as possible.

The early enzyme probes were based upon oxygen or ammonia specific electrodes. Updike and Hicks (1966) introduced the term enzyme electrode to describe a system using immobilized glucose oxidase in conjunction with a Clark type oxygen probe. In this case a reference probe was used to compensate for background fluctuations.

More recent developments have widened the range of reactions available.

Multiple enzyme systems have been coupled with the well defined glucose oxidase systems to allow detection of a range of compounds. Examples include sucrose (Satoh et al., 1976), lactose and maltose (Cordonnier et al., 1975). Other enzymes linked to oxygen probes include amino acid oxidase (Nanjo and Guilbault, 1974) and tyrosinase (Barker et al., 1974). An alternative to measuring the decrease in oxygen concentration associated with oxidase activity, is to measure the formation of hydrogen peroxide. Clark (1970) has described such a system for use with glucose oxidase. This approach has the advantage of being less sensitive to dissolved oxygen concentration in the medium.

The effects of contaminating catalase and peroxidase was overcome by the inclusion of inhibitors. Despite the advantages of the hydrogen peroxide method, Nanjo and Guilbault (1974) conclude that oxygen determination is superior on the grounds of sensitivity, reproducibility and simplicity.

Expansion of the linear concentration range for this type of probe can be obtained if oxygen is replaced with an alternative electron acceptor of greater solubility. Williams et al., (1970) demonstrated the use of benzoquinone as an electron acceptor in the glucose oxidase reaction, other acceptors proposed include, 2,6-dichloroindophenol sodium, methylene blue and pyrocyanin (Hoffman-La Roche, 1973). An alternative to replacement of the electron acceptor is indirect amperometric determination, Blaedel and Ohlsen(1964) followed the oxidation of potassium ferrous to ferricyanide while Pardue (1963) followed the oxidation of iodide to iodine. A similar amperometric determination has been used with a yeast lactate dehydrogenase. This enzyme, when

coupled with cytochrome b_2 has the advantage of having no requirement for NAD (Williams et al., 1970). The lactate to pyruvate equilibrium is more favourable than the NAD-dependent oxidation.

$$\text{L-Lactate}^{-1} + 2\text{Fe(CN)}_6^{-3} \xrightarrow{\text{LDH}} \text{Pyruvate}^{-1} + 2\text{Fe(CN)}_6^{-4} + 2\text{H}^+$$

$$2\text{Fe(CN)}_6^{-4} \xrightarrow{\text{P}^+} 2\text{Fe(CN)}_6^{-3} + 2\text{e}^-$$

Reactions have been based on other probes including pCO_2 (Guilbault and Shu, 1972), pH (Nilsson et al., 1973) and ammonium (Montvalvo and Guilbault, 1969. Montvalvo, 1970). Using variations on the above methods, in association with the specific ion probes available, a wide range of compounds can be determined.

Fundamental drawbacks associated with the use of selective ion probes can limit their usefulness in certain situations. Response time can be a major drawback associated with some systems. The methods of Tran and Brown (1975) quotes a time of 3 minutes to reach 99% of the steady state value, a figure which is not atypical, although response times of less than a minute have been quoted (Guilbault, 1973). The selectivity of some ion probes is such that other ions can interfere with the species being measured. The most dramatic example of this is the interference of monovalent cations with the response of the ammonium probe (Barker and Somers,1978). Although pH probes have been used in combination with enzymes, the associated problems are almost insurmountable, namely the requirement for the medium to have minimal buffering capacity and the non linearity of the response arising from the pH sensitivity of most enzymes.

These drawbacks have led to the investigation of alternative transducers for use with enzymes in specific monitoring devices.

Enzyme Thermistors

Immobilized enzymes have been increasingly used in thermal analyses during recent years (Spink and Wadsö, 1976). Early work was based on an immobilized enzyme reactor with a heat flow calorimeter (Johansson et al., 1973). Although such systems have excellent sensitivity the response time is slow (approximately 10 minutes).

Further developments along these lines led to the concept of the enzyme thermistor (Mosbach et al., 1975). In this device, an insulated immobilized enzyme column is constructed. A thermistor is mounted in the centre of the column of the packing material. This transducer is sensitive to extremely small changes in temperature, which are reflected in a changed impedence. Mosbach et al., found that in this type of reactor, approximately half of the heat evolved can be registered as a temperature change. Temperature changes between 0.004^{o} - $1^{o}C$ were observed and could be determined. The principle was demonstrated with glucose oxidase, urease and penicillinase. Subsequent work by the same group has demonstrated the feasibility of this system for on line control of an enzyme reactor through a PID controller (Dannielsson et al., 1979). The application for control of fermentations has also been studied (Nilsson et al., 1978).

The major drawback of the system is the necessity to minimise the fluctuations in background temperature, however, in their original

paper the authors conclude that a differential thermistor could improve sensitivity and reduce the effects of noise. In addition the use of buffers with high exothermic enthalpy of protonation could potentially increase the temperature changes in suitable systems (Mosbach et al., 1975. Johanson et al., 1973).

Fuel Cells

A recent development in enzyme technology is the use of biochemical fuel cells as sensitive detectors for a range of compounds. The concept of biological fuel cells in not new (Lewis, 1966), however, recent work with microbial methanol dehydrogenase has led to the development of a system capable of detecting a variety of primary alcohols in the range 10^{-7}- 5 x 10^{-11} moles (Plotkin et al., 1982). At present the method is not suitable for continuous analyses as it utilises soluble enzymes. Further work will be required using an immobilized enzyme to assess its true potential.

REACTORS

Chemical reactors can be classified by their flow characteristics into two ideal cases, well mixed and plug flow. In a well mixed system conditions will be uniform throughout the reactor, while plug flow assumes no axial dispersion (Lilly and Dunill, 1971). In practice, these ideal states will not be attained, and real systems will approach one or other extreme case more closely. Methods of analysing flow patterns within a reactor are well established (Danckwerts, 1953) and have been compiled (Cooper and Jeffreys, 1971). Lilly et al., (1966) applied these analyses to a biochemical reactor to confirm plug flow conditions in an immobilized enzyme column.

As the aim of this work is to study the utilisation of cofactors in an enzyme reactor, discussion will be centred on this area. Bright (1975) considered that in the near future, the only promising method of utilising coenzymes would be to delimit enzymes and derivatised coenzymes within a semi-permeable membrane. To some extent this picture has been changed by subsequent developments. The demonstration of enzyme-coenzyme complexes which have no external cofactor requirement (Mosbach , 1982) suggest that suitable complexes could be immobilized by attachment to insoluble supports for use in packed beds. This technique is still in its infancy and problems of cost and optimisation of the complex activity remain to be solved. Certainly for the present, Bright's comment would appear to be valid, that if continuous cofactor regeneration is required an ultrafiltration step is required. This is not to say that all components in the reactor would have to be soluble; either enzyme or cofactor should be soluble but not necessarily both.

Membrane Reactors

Membrane reactors may be operated in both homogeneous and heterogeneous mode, depending on flow conditions and enzyme loading used. Again these ideal states will not be attained and so overall reactor performance will result from the contribution of both systems.

Physical Characteristics

The physical characteristics of membrane reactors have been reviewed in depth and there is good understanding of the effect of the important operating parameters on overall reactor performance (Porter, 1972. Flaschel and Wandrey, 1979).

Ultrafiltration membranes are usually characterised in terms of their "nominal molecular weight cut-off". Essentially this means that the membrane should reject 90% of a globular protein of the cut-off weight. This cut-off figure can be correlated with the average pore size of the membrane, the flux through a single pore being described by the Hagen-Poiselle equation :-

$$J = \frac{K' r^2 . C_m . \Delta p}{8 . n . x}$$

J	$mol.cm^{-2}.s^{-1}$	mass flow
K'		constant of the component m (function of the rejection coeffecient)
r	cm^2	pore radius

C_m	$mol.cm^{-3}$	concentration of component m at membrane surface
Δp	$dyn\ cm^{-2}$	pressure drop across the membrane
n	$g.cm^{-1}.s^{-1}$	dynamic viscosity
x	cm	effective length of the pores

The rejection coefficient of the membrane for a given species for example, an enzyme, is defined :-

$$R = \frac{(E_R) - (E_F)}{(E_T)}$$

where :-

R		rejection coefficient
(E_R)	$mols\ cm^{-3}$	enzyme concentration in the reactor
(E_F)	$mols\ cm^{-3}$	enzyme concentration in the permeate
(E_T)	$mols\ cm^{-3}$	total enzyme concentration in the reactor at start of run.

The concentration profile of the enzyme in front of the membrane is a function of the rejection coefficient and can be expressed :-

$$(E_m) = (E_b).\ \frac{\exp\left(\frac{u.\delta}{D_e}1\right)}{R + 1(1-R).\ \exp\left(\frac{u.\delta}{D_e}1\right)}$$

where :-

(E_m)	$mols\ cm^{-3}$	concentration of enzyme at the membrane
(E_b)	$mols\ cm^{-3}$	concentration of enzyme in the bulk phase
u	$cm.s^{-1}$	linear velocity across the membrane (volumetric flow/unit area)

δ_1	cm	thickness of boundary layer
D_e	$cm^2.s^{-1}$	diffusion coefficient of the enzyme

This can be simplified in the case of no rejection to :-

$$(E_m) = (E_b)$$

and as previously stated to :-

$$(E_m) = (E_b).\ \exp\left(\frac{u.\ \delta_1}{D_e}\right)$$

if the species is totally rejected.

For homogeneous catalysis, the bulk concentration should be maintained as high as possible by minimising the concentration at the membrane.

Flaschel and Wandrey(1979) consider the practical factors affecting membrane reactors as follows: distribution of pore size, viscosity and effective pore length should be minimised. The pressure drop will be limited by the mechanical strength of the membrane. The linear velocity chosen will be a compromise between the problems of concentration polarisation and the requirement to optimise the productivity of expensive membranes. Control of concentration polarisation will depend on minimising the boundary layer, in addition to alterations in linear velocity across the membrane. This may be achieved by increasing the tangential flow across the membrane surface. Increases in the diffusivity of the protein would also be advantageous.

Both the above approaches depend on increasing the mass transfer of solute back into the bulk phase. Porter (1972) describes mass transfer in terms of the diffusivity and boundary layer height :-

$$K = \frac{D_e}{\delta}$$

where :-

K	cm s^{-1}	mass transfer coefficient
D_e	cm^2 s^{-1}	diffusion coefficient
δ	cm	boundary layer height

This can be rearranged to give :-

$$\delta = \frac{D_e}{K}$$

Diffusivity can be expressed by the Stokes-Einstein relationship :-

$$D_e = \frac{kT}{6\pi\mu\ r_p}$$

where:-

k	erg deg^{-1}	Boltzmann constant (molar gas constant divided by the Avogadro number)
T	^oK	temperature
μ	poise	viscosity
r_p	cm	radius of diffusing particle

From this relationship it can be seen that diffusivity can only be

increased by increasing the temperature or decreasing viscosity. As viscosity can not be decreased without altering the composition of the reactor feed, temperature is left as the only practical means of increasing diffusivity.

The relationship between shear and mass transfer in laminar flow required in thin channel reactors is based on the modified Graetz or Leveque solutions for convective heat transfer in laminar flow channels.

The general state can be expressed (Porter, 1972) :-

$$K = 0.816 \left(\frac{\gamma D_e^2}{L}\right) 0.33$$

where :-

K		mass transfer coefficient
γ		fluid shear
L	cm	channel length
D_e	$cm^2.s^{-1}$	diffusivity

Shear can be expressed :-

$$\gamma = \frac{6U}{b}$$

where :-

U	$cm^3.s^{-1}$	average fluid velocity
b	cm	channel height

Thus in a fixed bulk stream concentration flux should vary with the cube root of the wall shear per unit length.

Using these equations Porter (1972) found a good agreement between theoretical predictions and experimental results for ultrafiltrate flux. The experiments were carried out in spiral flow channel plates using albumin. Agreement was found to be within 25% for experiments carried out in the laminar flow region. The major deficiency in the theory is the need for experimental data to calculate the gel concentration of the protein. Gel concentration has been shown to vary in thin channel systems although the reason is unclear (Porter, 1972).

Kinetics

Study of the physical parameters of the reactor should allow an assessment of the contribution of both homogeneous and heterogeneous catalysis to overall reactor performance. The kinetics of enzymes in both continuous flow stirred tank reactors and packed beds have been described by Lilly et al., (1966) and Lilly and Sharpe (1968).

Immobilized enzymes in packed beds have been shown to follow an integrated form of the Michaelis Menten equation :-

$$S_o - S_t = K_2(E)_t + K_m \ln(S_t/S_o) \qquad (1)$$

where :-

S_o initial substrate concentration

S_t substrate concentration after time t

K_2 turnover number

K_m	equilibrium between enzyme, substrate and enzyme substrate complex
E	enzyme concentration
t	time

If plug flow is assumed, the residence time t in the bed is related to flow rate (Q) and void volume (V_1)

$$t = \frac{V_1}{Q} \tag{2}$$

Enzyme concentration can be expressed as total enzyme divided by total column volume

$$(E) = \frac{E}{V_{tot}} \tag{3}$$

Equations (2) and (3) can be substituted into equation (1).

$$(S_o - S_t) - K_m' \ln(S_t/S_o) = K_2(E/Q)(V_1/V_{tot}) \tag{4}$$

The term K_m' is an apparent value as immobilized enzymes may show altered kinetics.

Defining P as the fraction of substrate reacted,

$$P = (S_o - S_t)/S_o \tag{5}$$

and B, the voidage of the column,

$$B = V_1/V_{tot} \qquad (6)$$

allows simplification of equation (4) by substitution.

$$PS_o - K_m' \ln(1-P) = \frac{K_2EB}{Q} = C/Q \qquad (7)$$

where C is defined as the reaction capacity of the column in terms of substrate per unit time. The above equation can be rearranged to :-

$$PS_o = K_m' \ln(1-P) + C/Q \qquad (8)$$

This allows a plot of PS_o versus $\ln(1-P)$ to give a slope of K_m' and an intercept on the PS_o axis of C/Q.

For continuous flow stirred tank reactor the vessel is assumed to be ideally mixed (Lilly and Sharpe, 1968). This allows the Michaelis Menten equation to be expressed in terms of flow rate (Q) and reactor volume (U_1).

$$Q(S_o) = Q(S_i) + \frac{K_2(E)(S_i)U_1}{K_m + (S_i)} \qquad (9)$$

where :-

(S_i) substrate concentration in the reactor

The enzyme concentration may be defined as :-

$$(E) = \frac{E}{U_{tot}} \qquad (10)$$

where :-

E	total enzyme
U_{tot}	total reactor volume

Substitution of equation (10) in (9) with rearrangement gives :-

$$Q((S_o)-(S_i)) = \frac{K_2 E/U_{tot} U_1}{K_m/(S_i) + 1} \qquad (11)$$

The fraction of substrate reacted may be defined :-

$$P = 1 - (S_i)/(S_o) \qquad (12)$$

and the ratio of liquid to total volume

$$\epsilon = U_1/U_{tot} \qquad (13)$$

Equations (12) and (13) may be substituted into (11) to give :-

$$P(S_o)Q = \frac{K_2 E \epsilon}{K_m/(S_o)(1-P) + 1} \qquad (14)$$

This can be rearranged to allow a plot of $P(S_o)$ against $P(1-P)$:-

$$P(S_o) + P/(1-P)K_m = \frac{K_2 E \epsilon}{Q} = C/Q \qquad (15)$$

where:-

C	reaction capacity

For constant flow rates the plot should give straight lines with a slope of $- K_m$ and intercept of C/Q on the PS_o axis.

It must be stressed that both plots are based on the Michaelis Menten equation, therefore, care must be taken to ensure this mode of kinetics is obeyed by the system under study at steady state. For initial studies of complete systems it may be necessary to use numerical integration techniques to model the dynamic system up to the point where steady state is reached. The use of digital computers and dynamic simulation packages would appear to offer the most promising approach to predicting steady state performance of ultrafiltration reactor operating in a mixed mode.

This thesis reports the characterisation of an ultrafiltration reactor system with specific reference to the problems associated with ATP regeneration.

METHODS AND MATERIALS

ENZYME THERMISTOR

The thermistor design was based on that of Mosbach et al., (1975) with provision for an additional thermistor to allow a differential output, see Fig. 2.1. The sensor housing was constructed from acrylic plastic in the school workshops, couplings were threaded to take the Altex PTFE connectors, for compatability with the reactor system used. The design chosen allowed the complete thermistor housing to be submerged in a water bath for fine temperature control. Fig. 2.2 gives a diagramatic layout of the complete system.

The thermsitors used (R.S. 152 - 142) had a nominal resistance of 4.7 K Ω and were balanced in a bridge with resistors of 2.8 K Ω nominal value, see Fig. 2.3.

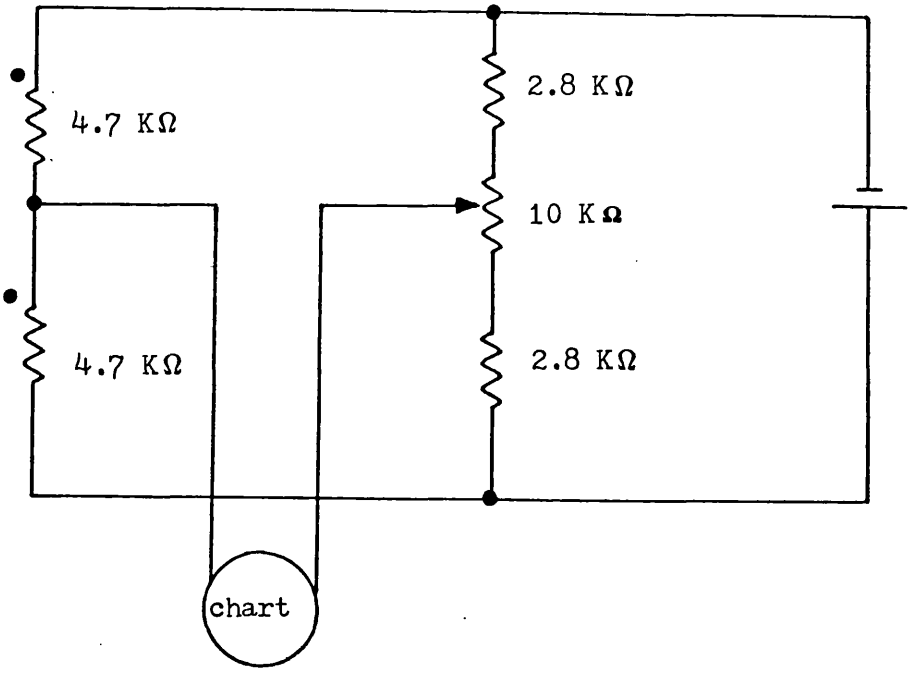


Fig. 2.3 Enzyme Thermistor Circuit Diagram

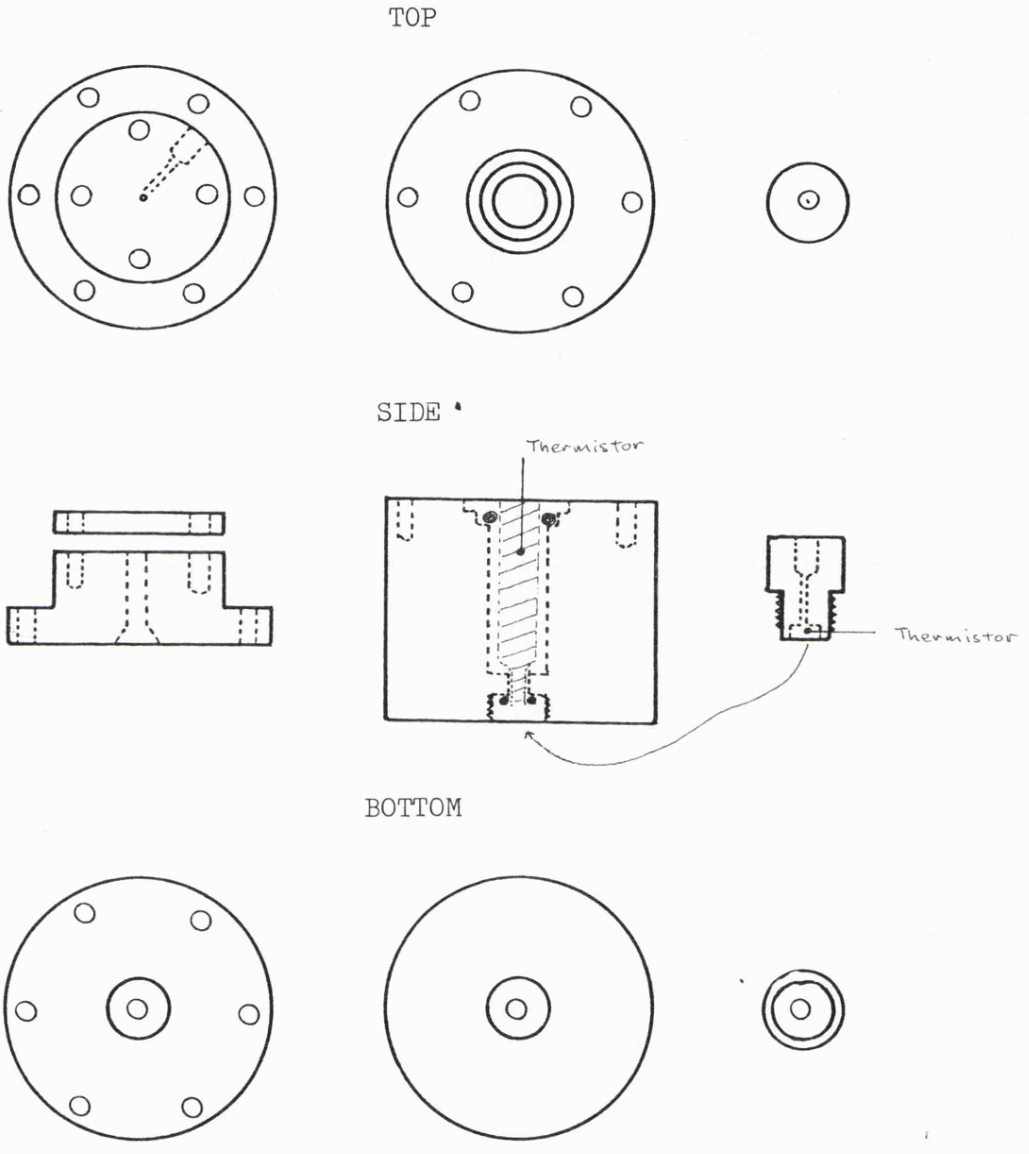


Fig. 2.1 Diagram of Thermistor Housing ($\frac{1}{2}$ scale)

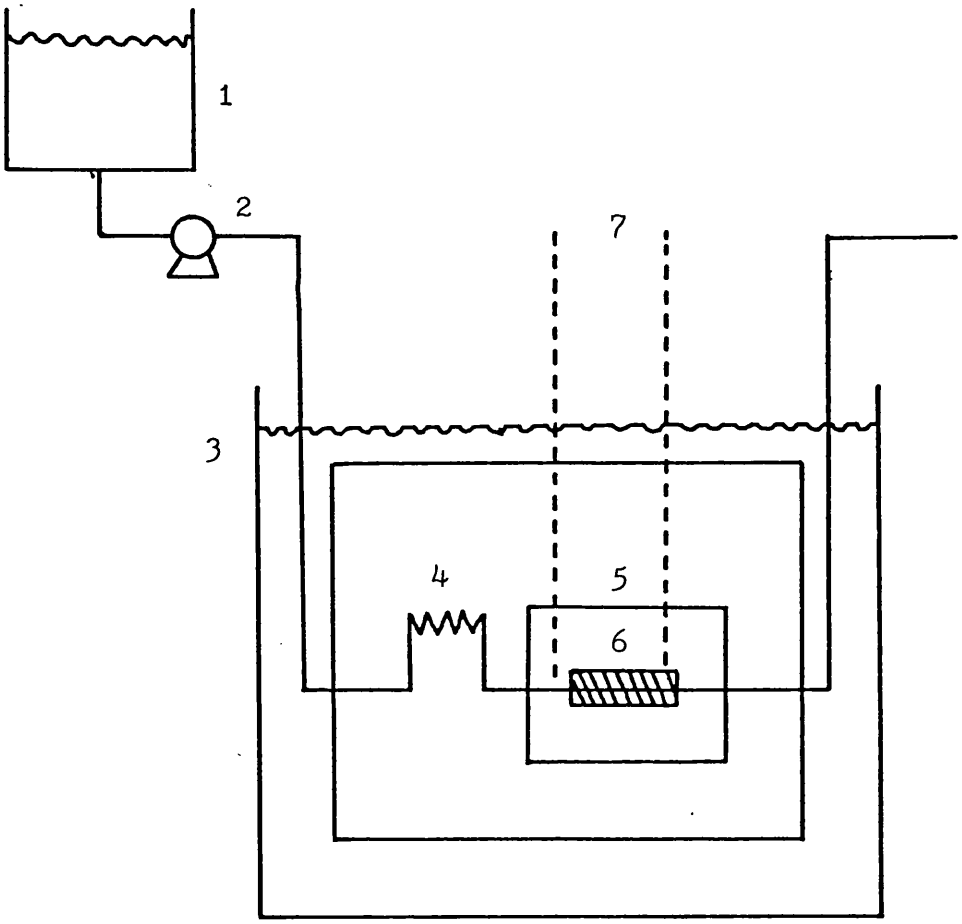


Fig. 2.2 Diagrammatic Layout of Enzyme Thermistor

1	Buffer reservoir	5	Perspex housing
2	Pump	6	Immobilized enzyme column
3	Water bath	7	Electrical connections
4	Heat exchanger		

The enzyme thermistors were driven using a constant voltage source and the output from the bridge was coupled to a high impedance chart recorder. Samples were pumped through the thermistor using a small peristaltic pump.

REACTOR STUDIES

Two reactor sizes were used throughout the course of this project. Both were constructed from acrylic plastic in the school workshops.

Studies of the physical parameters of thin channel ultrafiltration reactors were carried out in a system using 90 mm Amicon PM 10 membranes (Fig. 2.4). Total reactor volume including recycle was 50 cm^3 and exposed membrane area was 20.6 cm^2. All connections were made using 4 mm to 1/8" b.s.p. thread nylon compression couplings and 4 mm internal diameter nylon pressure tubing. The membrane was supported with a perforated stainless steel plate. A line diagram of the complete system including monitoring equipment is given in Fig. 2.5.

The second reactor also a thin channel device was based on 43 mm diameter membranes. The volume of this system was minimised to reduce the use of expensive reagents in catalytic studies. All connections were made using Altex PTFE connectors (1.5 mm to 1/8" n.p.t.) with 0.8 mm internal diameter tubing, see Fig. 2.6. The complete system was similar to Fig. 2.5 but without provision for pressure monitoring.

All reactor experiments were carried out using micrometering pumps to ensure constant accurate flow rates at all experimental pressures. Pumps were calibrated against a reference pressure of 15 psi using a pressure relief valve. All pump components in contact with liquids were either ceramic or stainless steel, all seals were PTFE.

Pressure was continuously monitored using a pressure transducer calibrated against a reference pressure gauge. The pressure transducer

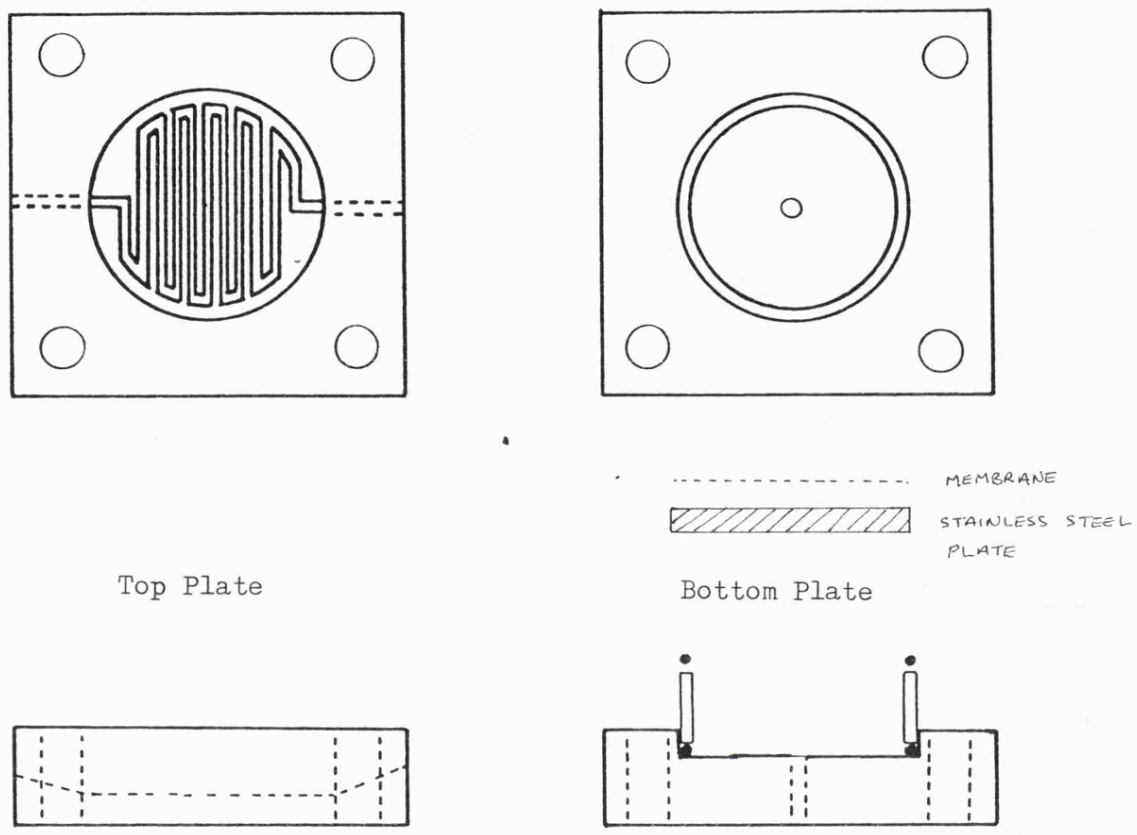


Fig. 2.4 Diagram of Large Reactor ($\frac{1}{3}$ scale)

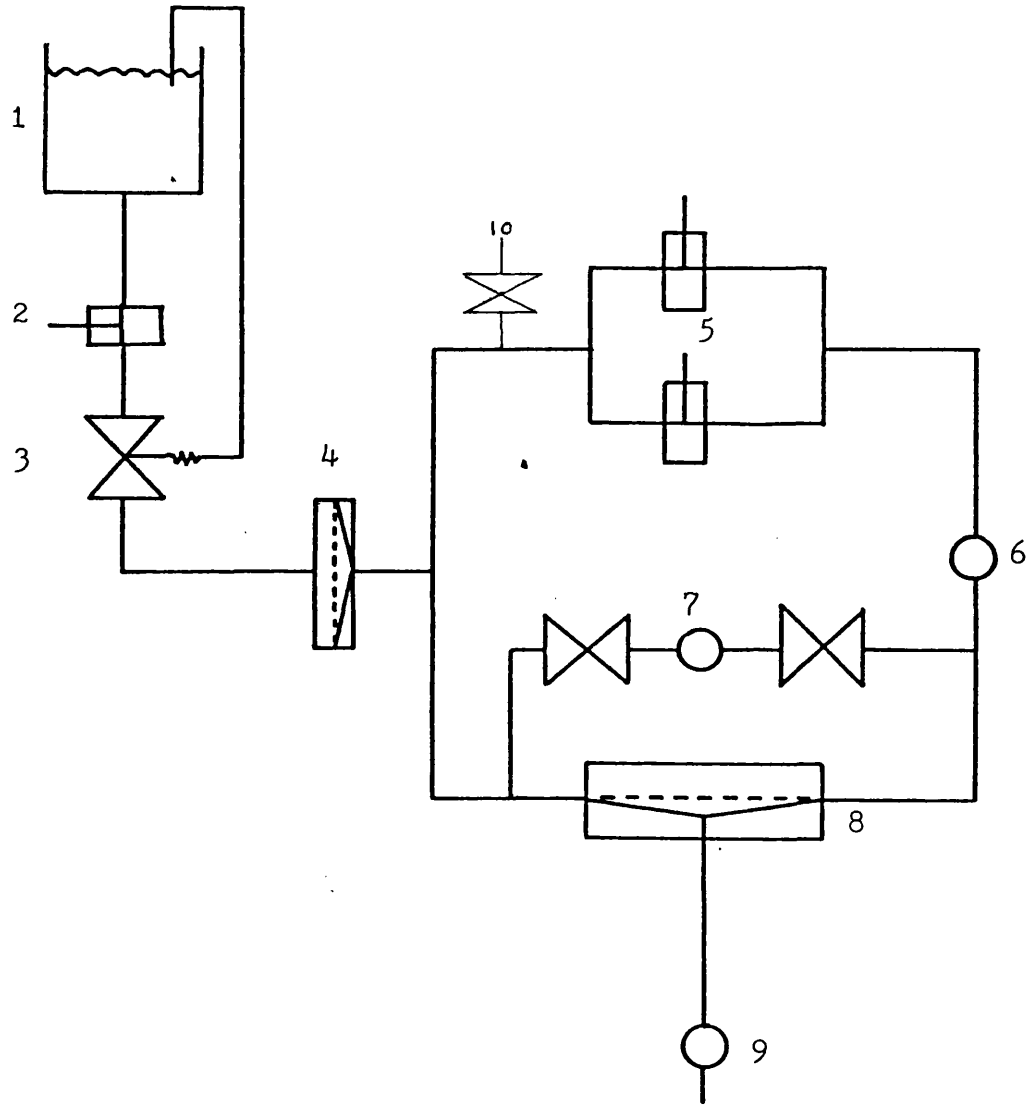


Fig. 2.5 Diagrammatic Layout of Reactor Systems

1	Substrate reservoir	6	Thermistor
2	Feed pump	7	Pressure transducer
3	Pressure relief valve	8	Membrane housing
4	Prefilter	9	pH probe
5	Recycle pumps	10	Injetion port

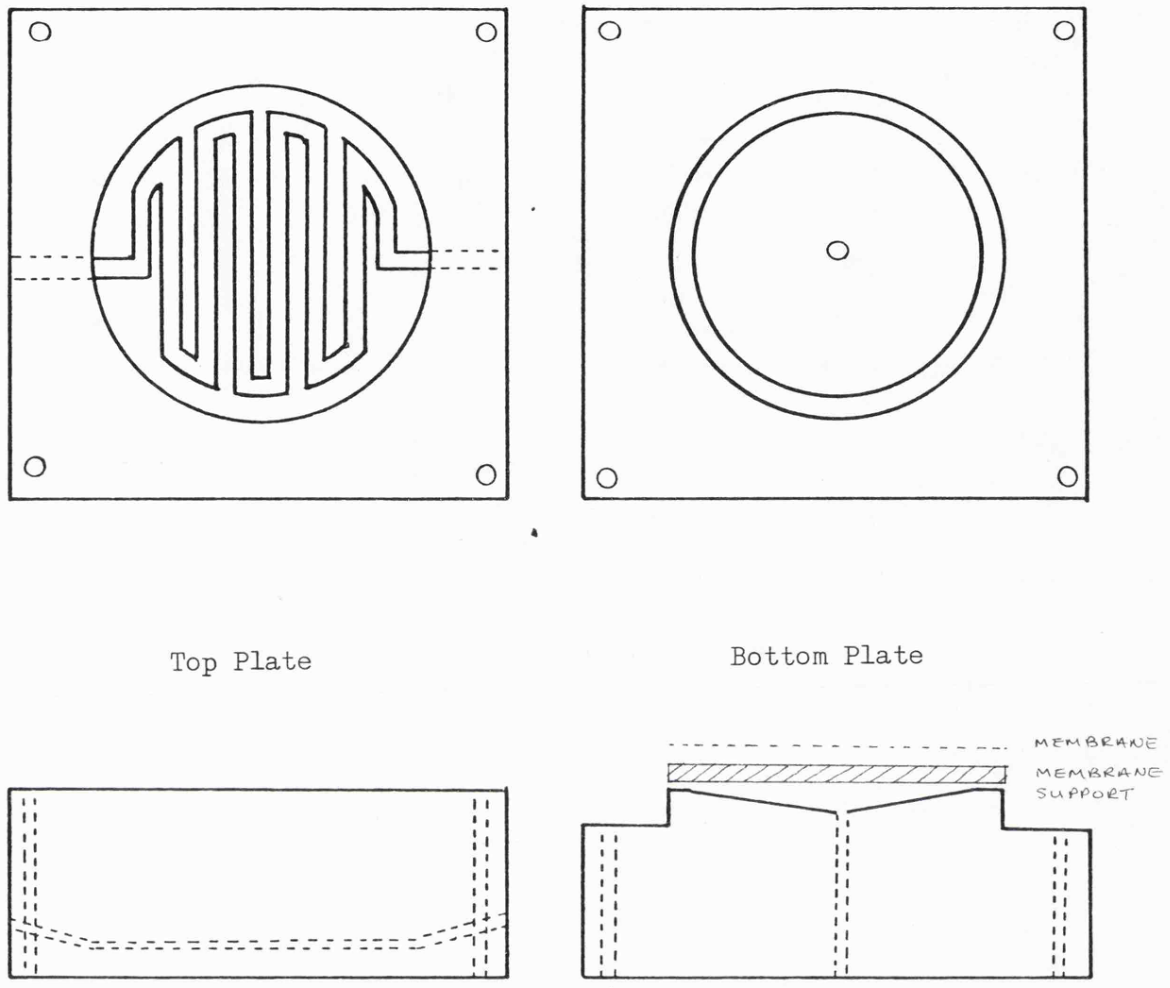


Fig. 2.6 Diagram of Small Reactor (scale 1:1)

was constructed from stainless steel and comprised two strain gauges attached to a stainless steel diaphragm. The built in wheatstone bridge circuit required 10 volts d.c. excitation and gave an output of 0 - 50 mV linearly related to absolute pressure. Temperature was monitored using a thermistor device driving a high impedence chart recorder through a wheatstone bridge.

ENZYME ASSAYS

Enzyme concentrations were adjusted such that doubling the concentrations would double the rate at the maximum cofactor concentration. Linking enzymes were present in two fold excess to minimise lag times. Conditions were adjusted such that ATP/ADP was rate limiting in all assays.

Hexokinase (EC 2.7.1.1 ATP: D-hexose 6-phosphotransferase)

Hexokinase was assayed by coupling the reaction to glucose 6 phosphate dehydrogenase, and observing the increase in E_{340} in a Cecil 272 spectrophotometer at 30 °C. The assay mixture, final volume 1 ml, contained 100 µmol D-glucose, 2 µmol NAD, ATP, 35 µg hexokinase, 50 µg glucose 6 phosphate dehydrogenase.

All solutions were made up in 25 mMol tris buffer, pH 7.7 containing 50 mM magnesium chloride.

Acetate Kinase (EC 2.7.2.1. ATP: acetate phosphotransferase)

The phosphorylation of ADP was linked to the reaction with hexokinase and glucose 6 phosphate dehydrogenase. The conditions were as for the hexokinase assay, in addition the mixture contained 50 µmol acetyl phosphate, ATP was replaced with ADP. The reaction was started by the addition of 1.2 µg acetate kinase.

Determination of enzymically active bound cofactor was carried out in

1 ml containing 10 μmol acetyl phosphate and varying concentrations of free and bound ADP. The reaction was started by the addition of 12 μg of acetate kinase. All solutions were made up in 75 mM tris pH 7.5, containing 110 mM magnesium chloride. The reaction was allowed to proceed for 30 minutes at 25 °C and then was stopped by boiling for 3 minutes. ATP formed was determined using the hexokinase assay as described above. Control experiments showed that ATP exposed to 100 °C for 3 minutes showed no detectable breakdown.

Pyruvate Kinase (EC 2.7.1.40 ATP: pyruvate phosphotransferase)

Pyruvate kinase was assayed by coupling to lactate dehydrogenase and observing the increase in E_{340} in a Cecil 272 spectrophotometer. The assay was a modification of the method as specified in Bergmeyer (1974). The mixture, final volume 1 ml, contained 0.5 μmol potassium chloride , 20 μmol magnesium sulphate, 0.5 μmol phosphoenolpyruvate, 0.1 μmol NADH and ADP. The reaction was started by the addition of 6.7 μg lactate dehydrogenase and 5 μg pyruvate kinase. All solutions were made up in 80 mM tris pH 7.0.

Myokinase (EC 2.7.4.3 ATP:AMP phosphotransferase)

This method was again based on the hexokinase glucose 6 phosphate dehydrogenase assay. Conditions used were similar, with the exception that ATP was replaced with ADP and 50 μg of myokinase was used to start the reaction.

CHEMICAL ESTIMATIONS

Total Phosphate

Total phosphate was estimated by the method of Bartlett (1959) using sodium dihydrogen orthophosphate as a standard.

Dextran

Dextran was estimated using the method of Nelson (1944). Standard curves were constructed using glucose and dextran T40 as reference.

BIOCHEMICAL ESTIMATION

Glucose 6 Phosphate

Reactor effluent was assayed for glucose 6 phosphate using glucose 6 phosphate dehydrogenase. Where necessary samples were diluted with 25 mM phosphate buffer, pH 7.5 (as used in the reactor). The reaction mixture was made 14 mM with respect to NAD^+ and the reaction was started by the addition of 5 μg glucose 6 phosphate dehydrogenase. The initial rate was followed by the change in E_{340}. The amount of glucose 6 phosphate was quantified from a standard curve of rate against concentration.

ACTIVATION OF SEPHAROSE 4B

Sepharose 4B was activated with cyanogen bromide by the method of Larson and Mosbach (1974). 5 grams of dextran was dissolved in 50 ml water. The reaction was started by the addition of 250 mg cyanogen bromide. Throughout the course of the reaction pH was maintained at 10.8 by the addition of 1 M sodium hydroxide. When consumption of base was complete, the pH was adjusted to 8.5 with 0.1 M hydrochloric acid.

SIMULATIONS

Dynamic computer simulations of reactor performance were carried out using the Continuous Systems Simulations language version IV.

See Appendix 1.

MATERIALS

All general laboratory reagents were purchased from BDH Chemicals Ltd. Analar grade was used throughout, where possible. Specialised chemicals and equipment were obtained from the following sources :-

Aldrich Chemical Co. Ltd. (Gillingham, Dorset.)
1,-4-butanediol diglycidyl ether (Technical)

Amicon Ltd. (High Wycombe, Bucks.)
PM 10, PM 30 ultrafiltration membranes.

Anachem Ltd. (Luton, Beds.)
1.5 mm to 1/8" n.p.t. PTFE connectors.

BDH Ltd. (Poole, Dorset.)
Spherical hydroxyl apatite
'Aristar' urea
Bromocresol green

Boehringer Corporation (London) Ltd. (Lewis, Sussex).
Urease 'Enzygel'
Hexokinase
NADH

Bell and Howell Ltd.
Pressure transducer 0-6 Bar type

Circlex Ltd. (Fleet, Hants.)

4 mm 1/8" b.s.p. nylon compression joints

6 mm $\frac{1}{4}$" b.s.p. nylon compression joints

4 mm and 6 mm i.d. nylon tubing

Pharmacia (U.K.) Ltd. (London)

Dextran T40

Sephadex G25 (fine)

Sepharose 4B

R.S. Components Ltd. (London)

Thermistors type 152 - 142

All other electrical components

Sigma London Chemical Co. Ltd. (Poole, Dorset)

All biochemicals not previously specified

Procion dyes courtesy of I.C.I. Organic Ltd.

RESULTS

ENZYME THERMISTOR

Previously reported enzyme thermistor systems have required sophisticated temperature control to eliminate the effects of background temperature fluctuations. In addition, these systems have used covalently bound or physically entrapped enzymes, requiring the system to be dismantled when the enzyme has to be replaced.

To overcome these drawbacks, the use of differential thermistor arrangement with an adsorption matrix has been studied.

Physical Characteristics

The success of this type of detector depends on a temperature increase sufficient to be measured. The relationship between temperature rise and energy input is based on the thermal capacity of the column in the vicinity of the thermistor. The sensitivity can be measured by inputting a known amount of energy and monitoring the temperature rise.

The reaction column was packed with an inert support (Sepharose 4B) in which a 1 KΩ resistor was implanted. The complete thermistor housing was placed in an unthermostated water bath and allowed to equilibrate for 12 hours.

The response of the thermistor to a given temperature change can be calculated by the following equation (R.S. Data Sheet R/1867, 1977) :-

$$R_2 = R_1 . e^{\left(\frac{B}{T_2} - \frac{B}{T_1}\right)} \qquad (1)$$

where :-

B	^oK	characteristic temperature constant
T_1, T_2	^oK	bead temperature
R_1		resistance of thermistor at T_1
R_2		resistance of thermistor at T_2
e		2.7183

For a given resistance change as described above the output of the thermistor bridge circuit can be calculated using the following equation :-

$$\Delta v = \frac{R_1}{R_1 + R_2} - \frac{R_1 + \Delta R}{R_1 + \Delta R + R_2} . v' \qquad (2)$$

where :-

Δv	volts	output voltage change
R_1		resistance of thermistor 1
R_2		resistance of thermistor 2
v'	volts	excitation voltage
ΔR		change in resistance of thermistor 1

The excitation voltage applied to the bridge circuit is constrained by the maximum power rating of the thermistor used. Within this constraint the higher the excitation voltage, the greater the sensitivity of the detector. The simple relationship between current, voltage, resistance and power are as follows :-

$$w = \frac{V^2}{R} \qquad (3)$$

where :-

w watts power

When the arms of the bridge are balanced, V is equal to half the excitation voltage.

Using these equations the maximum possible excitation voltage can be calculated given the power maximum of the thermistor (RS 151-142 = 70 mw). In practice, however, it may be advantageous to operate at a lower voltage to minimise the effects of energy dissipated from the thermistor, also the working life of the component will be shortened if operated at its maximum rating.

From the above theoretical considerations it was decided to use an excitation voltage of 2.5 volts. This meant the thermistor would be operating at 0.5% of its maximum power rating. Theoretical calculations based on the quoted characteristics of the thermistors used suggested a response of 2.25 mv per 0.1 ^{o}C should be obtained.

A study of energy input against $\hat{}_{peak}$ output voltage is shown in Fig. 3.1. The results were obtained using a liquid throughput of 20 ml/hour and were measured on a chart recorder sensitivity of 0-5 mv full scale deflection. Known quantities of energy were input by passing a known current through the 1 KΩ resistor. Energy values of 60 mJ applied over a period of 1 minute can be reproducibly measured and did not represent the limits of sensitivity for the system. The tailing off of the response at higher energy inputs was shown to result from the

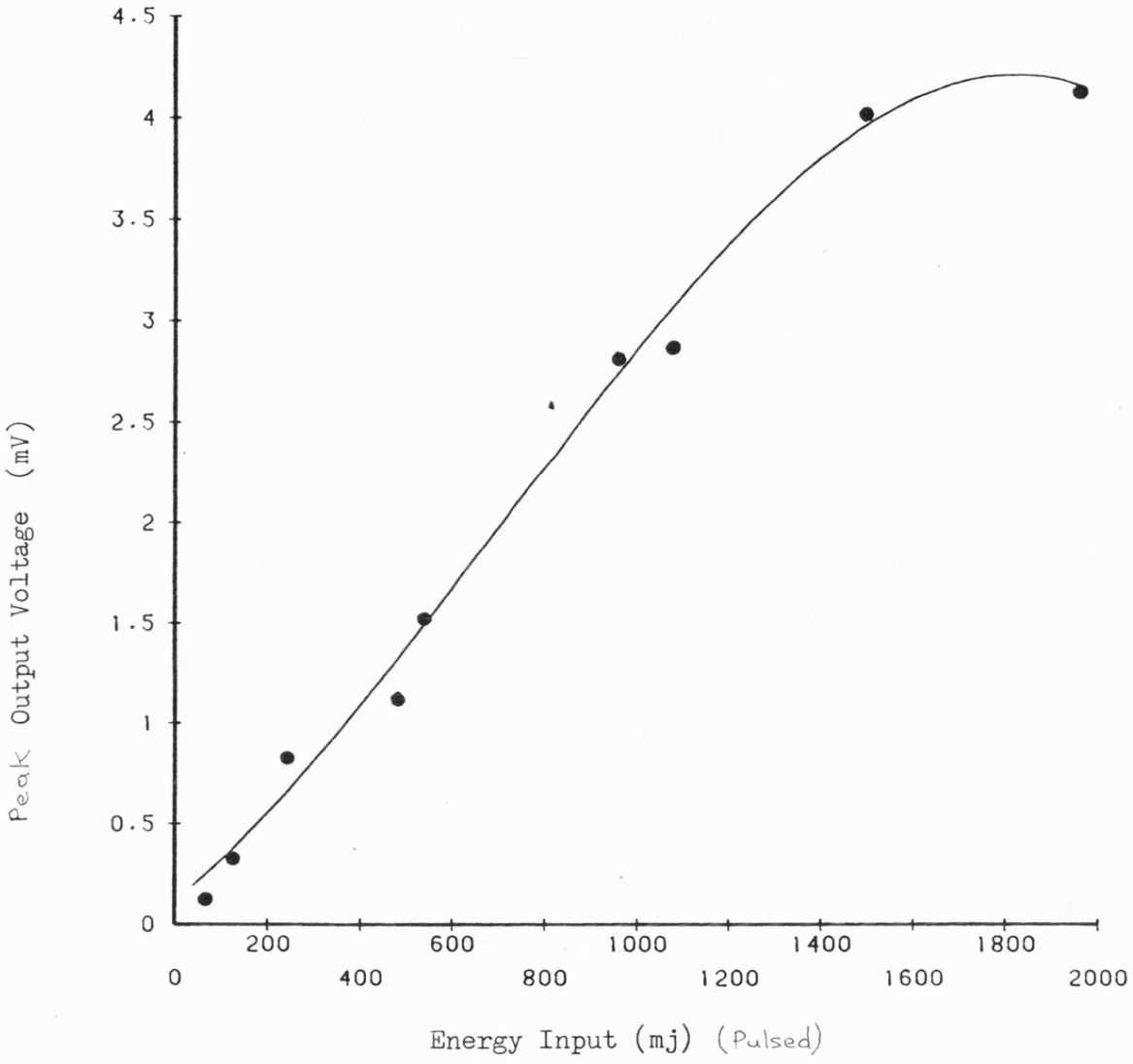


Fig. 3.1 Thermistor Sensitivity

increased time taken to reach steady state. Typical results are shown in Table 3.2 for a continuous constant energy input, until steady state is reached.

Table 3.2

mw input	time to reach steady state
1	5 min
2	17 min
4	34 min
9	44 min

The time taken to reach steady state reflects the heat transfer limitations from the epoxy encased resistor. The effect of volumetric flow rate on time taken to reach steady state can be seen in Table 3.3.

Table 3.3

mw input	flow rate (mls/hour)			
	20	30	60	
1	5	3.6	2.4	(minutes)
2	17	8.4	3.5	(minutes)

The effect of flow rate on peak height sensitivity can be seen in Fig. 3.4 for two energy input values. It was found to be impossible to obtain reasonable base lines at flow rates less than 20 ml/hour. This is to be expected from the differential nature of the circuit. As the flow rate approaches zero, the sensors can be considered to be in unrelated

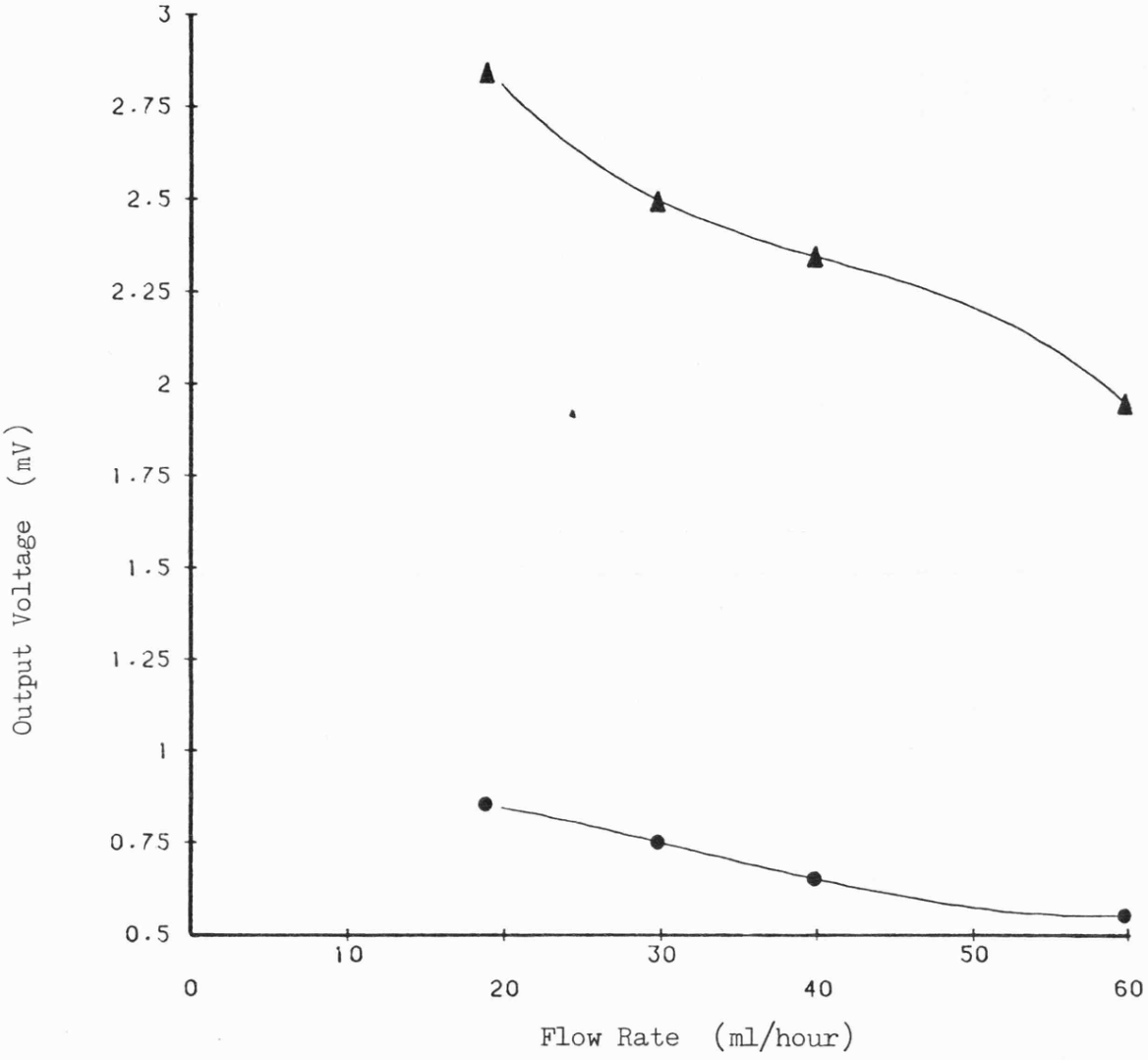


Fig. 3.4 Effect of Flow Rate on Sensitivity

● —— ● 240 mj Pulsed input

▲ —— ▲ 960 mj Pulsed input

environments and the advantages of the reference sensor are lost.

Previous workers have calibrated their systems by calibrating the response of the thermistor in an accurately controlled temperature bath. This approach is simple to employ in the case of a single thermistor detector but is more complex in the differential system used here. The circuit used can be seen in Fig. 2.3. The potentiometer is used to control the output voltage by balancing the resistance on both arms of the bridge. If T_1 is the thermistor in the bed, the effect of a change in resistance can be expressed by equation 2.

This theoretical prediction of the response of the circuit gives a general guide to its sensitivity but cannot be used to give exact values as the two thermistors will not be exactly matched in response. Care was taken to pick closely matched components but response curves vary. However, as the range of interest is less than 1 ^{o}C, the percentage errors introduced by such discrepancies will be insignificant in the case of experimentally obtained data.

One other aspect of the thermistor response curve which has significance is the tailing off at high temperatures. From theoretical considerations an increase of 20 ^{o}C in the operating temperature for the thermistor would account for a decrease of 12.5% in the sensitivity. The effect of temperature is shown in Fig. 3.6.

Enzyme Studies

Preliminary enzyme studies were carried out using commercially

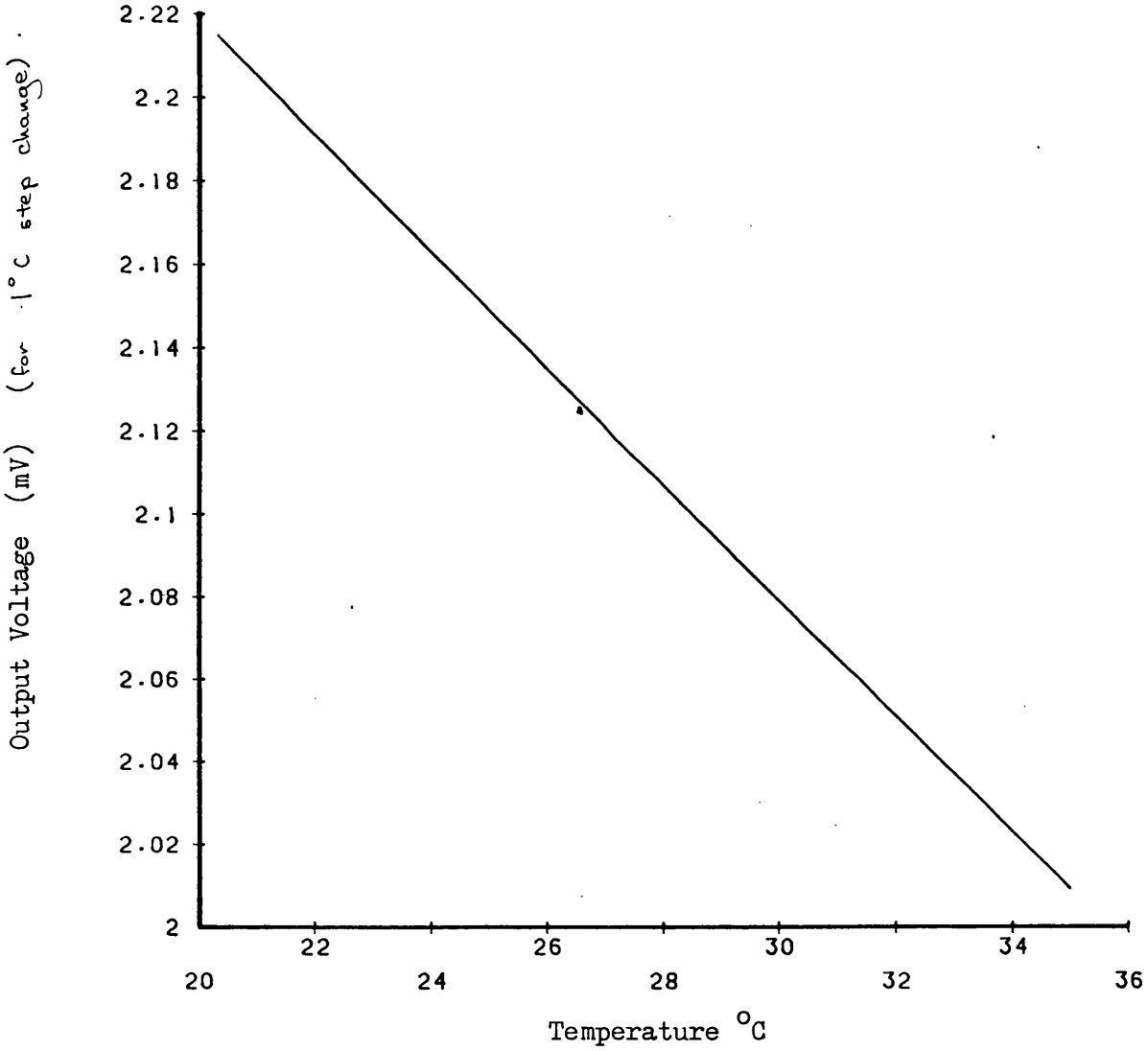
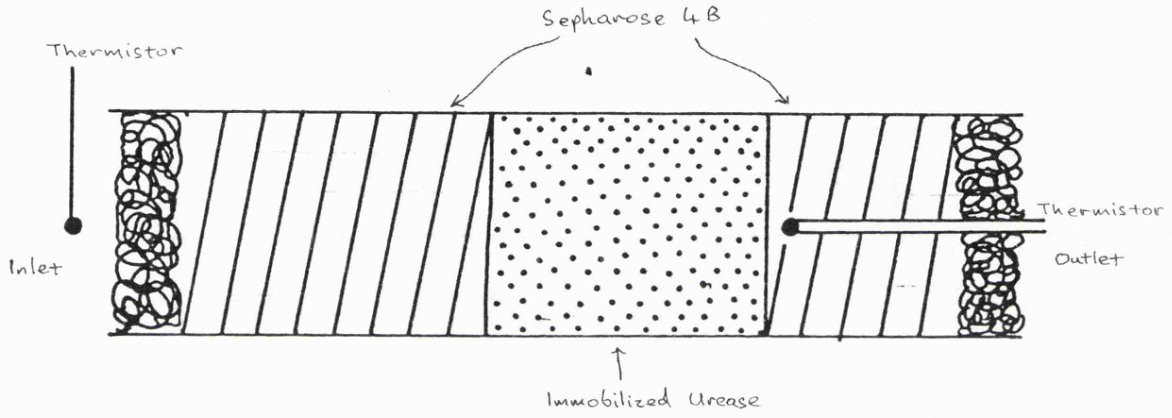


Fig. 3.6 Theoretical Plot of Thermistor Sensitivity against Ambient Temperature

available immobilized urease. This enzyme was chosen because of its availability and also as its use in enzyme thermistor devices has been previously documented (Mosbach et al., 1975). The immobilized enzyme (100 mg) was supported on a layer of inert Sepharose 4B in the reactor column. The column thermistor was positioned at the down stream edge of the immobilized enzyme and a further layer of Sepharose 4B was added.



Positioning of Urease in the Column.

The complete thermistor assembly was allowed to equilibrate for twelve hours with buffer (0.1 M potassium phosphate, pH 7) passing through.

The excitation voltage was 2.5 volts with a flow rate of 19 ml/hour. The chart recorder sensitivity was 0 - 2 mv full scale deflection, with a chart speed of 3 cm/hour. All measurements were taken in an unthermostated water bath at ambient temperature (approx. 20 ^{o}C). All

samples were added in 0.5 ml aliquots using solution at a range of concentrations.

The response of the thermistor to varying amounts of urea can be seen in Fig. 3.7. The non linear response shown would be expected from an examination of the Michaelis constant of urease for urea. Reithel (1971) quotes a value of 19 mMol, thus the reaction is becoming saturated at the higher concentrations. The non linear response causes calibration problems if extended concentration ranges are to be studied.

In practice stable base lines could be obtained after approximately 24 hours with buffer throughput. Fig. 3.8 shows a trace obtained after this time.

Use of an Adsorption Matrix

Having demonstrated the feasibility of the system using a covalently bound enzyme, an attempt was made to use an adsorbed one. Early experience showed the position of the thermistors to be critical, hence setting up the detector could be time consuming. The use of an adsorption matrix allows the potential to reload the column in situ, thus avoiding the need to dismantle the detector and minimising the time required for temperature equilibration after reloading.

The adsorption matrix chosen was a stablilzed form of hydroxylapatite. Hydroxylapatite is a form of calcium phosphate having a large crystalline structure. Its use for protein adsorption has been widely documented. The major disadvantage of hydroxlapatite is its poor

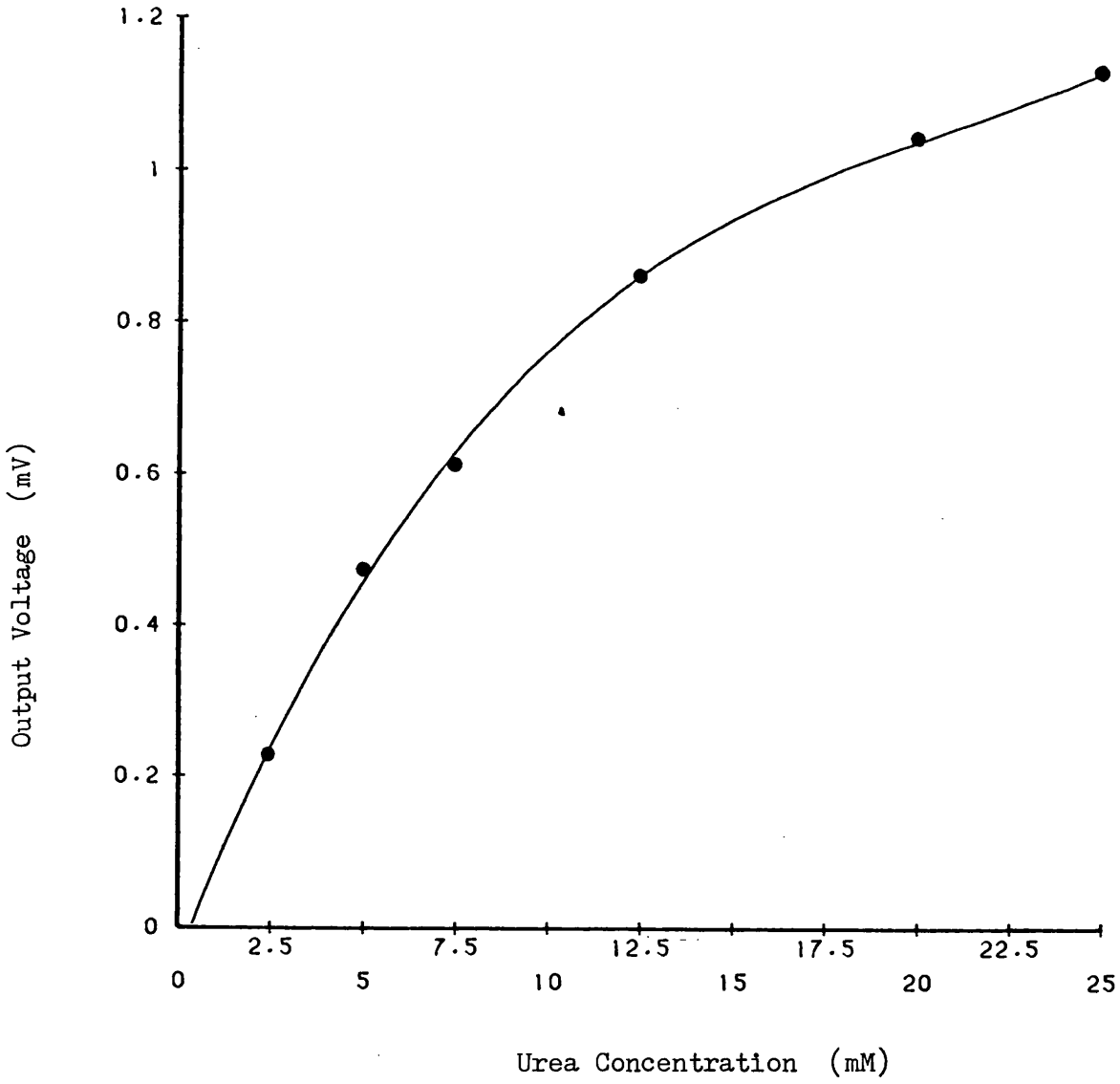


Fig. 3.7 Calibration Curve for Urease Column

(≃20 °C Flow rate 19 ml/hour)

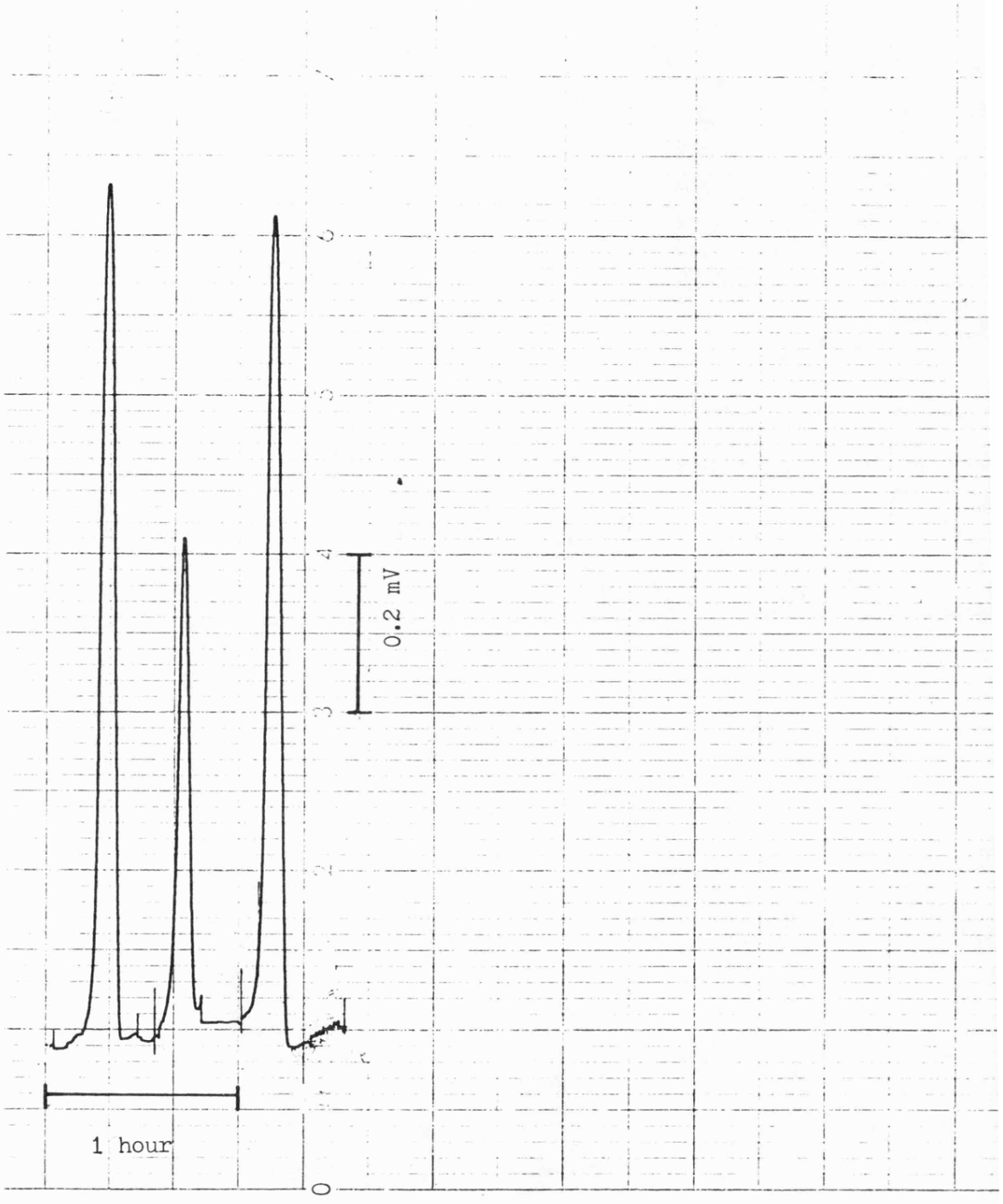


Fig. 3.8 Trace obtained Urease Column

($\simeq 20\ ^{o}C$; Flow rate 19 ml/hour)

mechnical stability. This leads to the formation of fines and reduced flow rates in column systems. These disadvantages have been overcome by the production of a stabilised beaded form of the material (B.D.H. Spheroidal hydroxylapatite). Although the adsorption capacity of this material is lower than the crystalline form it has good mechanical stability and allows the use of high flow rates.

The column was packed with 1 ml of spheroidal hydoxylapatite particles supported between layers of Sepharose 4B as previously discussed. The column was equilibrated overnight at ambient temperature. The enzyme system chosen for this study was glucose oxidase/catalase. This choice arose from the availability of highly active commercially available preparations of the enzymes and the favourable ΔH value of the reaction (Catalase, -100.4 KJ mol^{-1}; Glucose oxidase, -80 KJ mol^{-1}; Mosbach and Danielsson, 1981).

The enzyme was loaded onto the column by pumping a solution through at a low flow rate, approximately 10 ml/hour. The amount of enzyme bound was estimated by measuring the OD_{280} before and after passing through the column. Typically it was possible to bind 0.2 mg in both 10 mM potassium phosphate and 10 mM tris buffers both at pH 7.0.

The response of the system to various sized aliquots of glucose solution at a range of flow rates was studied. Unfortunately, the results were complicated by a large decrease in temperature after the initial increase (Fig. 3.9). This was found at all conditions tried.

Examination of the literature suggested that this change may well be

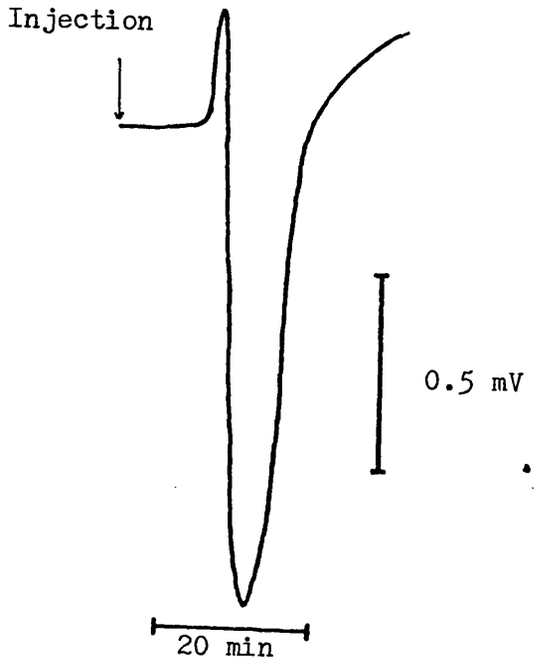


Fig. 3.9 Response of Glucose Oxidase/Catalase Column to 10 mM Glucose (Sample size, 0.5 ml)

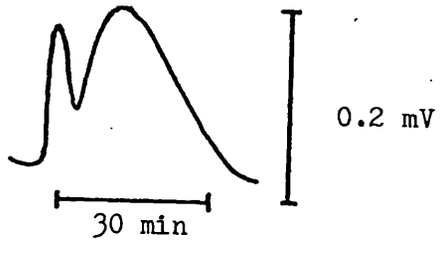


Fig. 3.10 Trace Obtained with 0.1 M Sodium Hydroxide Wash

accounted for by the enthalpy of adsorption if the glucose was being adsorbed to the column

Attempts to substantiate this assumption by saturating the column with a high concentration of glucose was inconclusive but an increase in temperature was noticed when the column was washed with 0.1 M sodium hydroxide prior to reloading , see Fig. 3.10. This result suggested that the amount of enzyme adsorbed to the column may be shown by an apparent change in temperature. In fact no apparent temperature change was noticed, however, this may be expected from the small mol fraction involved and the period of time over which the protein was adsorbed. The double peak seen in Fig. 3.10 may result from protein and glucose sequentially desorping. The effect would be more noticeable because of the shorter time scale.

REACTOR STUDIES

Previous work by Gacesa (1977) has shown that the ultrafiltration reactors used in this study operate essentially as well mixed systems. However, the analysis of residence time distribution was carried out with a low molecular weight tracer not susceptible to concentration polarisation.

The situation in the presence of macromolecules is less clear. Gacesa found enzyme inactivation followed two first order rates. One rate was found to be associated with enzyme deposition on the membrane; the other was attributed to the effects of shear. Subsequent work by Virkar et al; (1981) has shown enzymes unaffected by shear stresses normally encountered in mixing and pumping operations. The work of Greco et al., (1979) has shown that enzymes polarised onto ultrafiltration membranes are still active but are affected by mass transfer limitations of the substrate through the gel layer.

It would seem necessary to undertake a more detailed study of reactor performance in view of these findings.

Attainment of Steady State

The time taken for transmembrane pressure drop to reach steady state value was measured in buffers of two ionic strengths. All other conditions were identical. The plots of pressure against time can be seen in Fig. 3.11.

This experiment was repeated in distilled water to accentuate the effect;

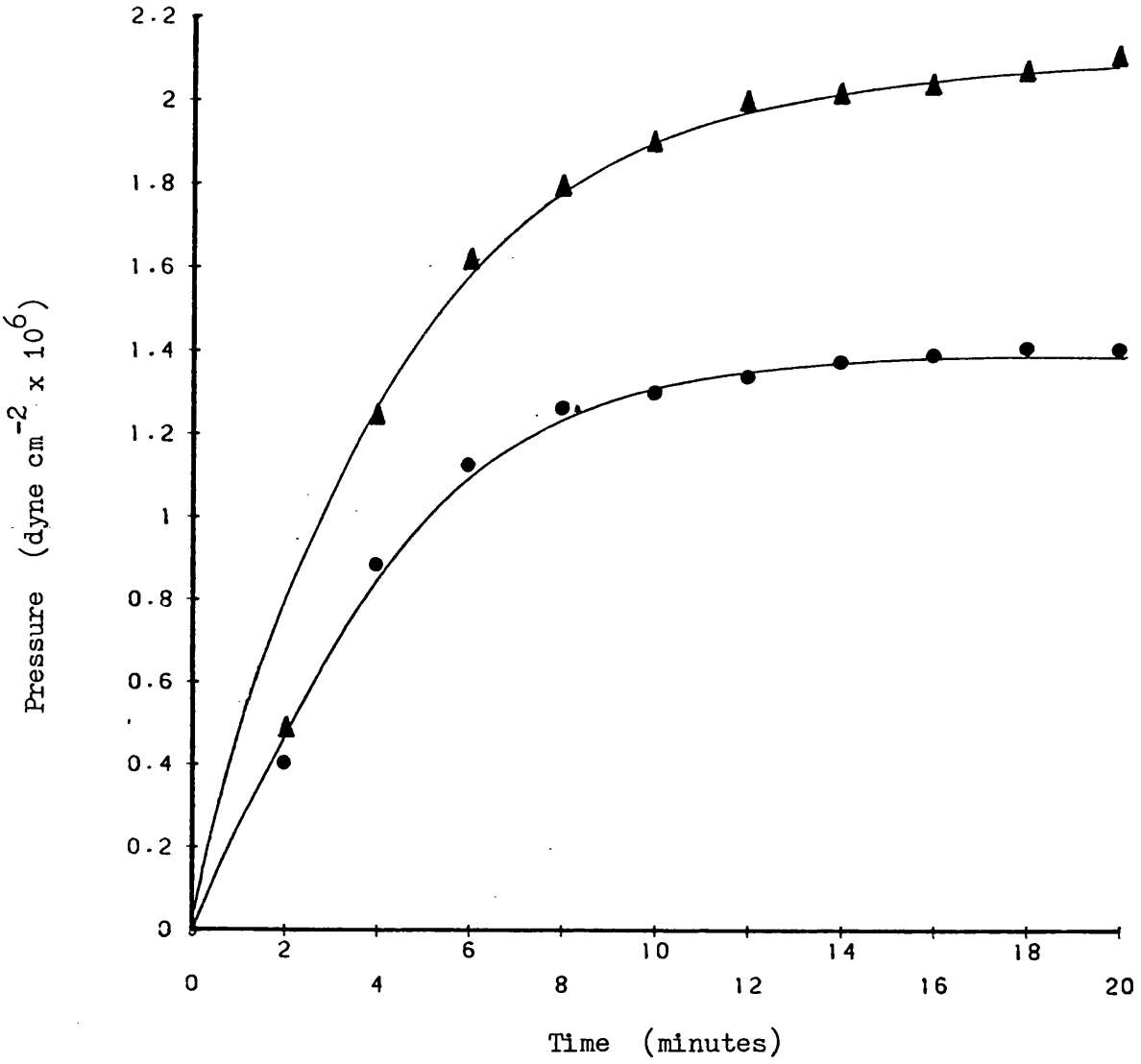


Fig. 3.11 Attainment of Steady State

(20 mg BSA (Bovine Serum Albumin), Specific Flux 8.76 x 10^{-3} cm s^{-1}, Volumetric recycle, 0.54 ml sec^{-1})

● —— ● 100 mM sodium bicarbonate buffer pH 9.1

▲ —— ▲ 10 mM sodium bicarbonate buffer pH 9.1

however, the pressure drop showed a continued increase up to the limits of the reactor. This was consistent with precipitation of the protein. These results suggest that at buffers of low concentration, protein precipitation or adsorption to the gel layer may account for the secondary decay rate.

The Effects of Transmembrane Flux and Protein Concentration on Concentration Polarisation

The gel polarisation model has been widely used to explain the relationship between flux, mass transfer and protein concentration in ultrafiltration systems. The model was originally proposed to describe separation systems where a constant pressure was applied as a driving force and the resultant flux was dependent on the gel layer formed at the membrane surface. In this analysis, the flux is independent of driving force as the gel resistance self adjusts until at steady state the convective transport is balanced by the back diffusion (Porter, 1972).

$$JC = D.\frac{dc}{dx} \qquad (1)$$

where :-

J	solvent flux
C	concentration of membrane retained species
D	diffusivity
$\frac{dc}{dx}$	concentration gradient in front of the membrane

The assumption inherent in the gel polarisation model, namely that gel concentration is constant, allows this equation to be integrated to

give :-

$$J = \frac{D}{\delta} \ln \frac{C_G}{C_B} \qquad (2)$$

where :-

D	diffusivity
δ	the boundary layer over which the concentration varies
C_B	the bulk concentration of membrane retained species
C_G	the gel concentration of membrane retained species

The term D/δ has been shown to be equivalent to the mass transfer coefficient for a large number of colloidal particles and macromolecules.

This model allows prediction of flux rates from a system having a known mass transfer coefficient. As the bulk concentration is known, the gel concentration can be used to solve for J. The model requires the assumption that similar macromolecules have a constant gel concentration. In practice, however, it is necessary to calculate the value from experimental results as the gel concentration has been shown to vary with reactor configuration (Porter, 1972).

The situation in the case of ultrafiltration reactors is somewhat different. The reactor would ideally be operated at a constant flux. This situation could be considered as the reverse of the previous system i.e. flux is constant and determines the concentration of macromolecule at the membrane.

If the gel polarisation equation (1) is integrated with the boundary condition :-

$$x = \delta \, , \; c = C_b$$

an equation of the same form as 2 is obtained

$$J = \frac{D}{\delta} \ln \frac{C_m}{C_b} \qquad (3)$$

in this case the term C_m refers to the concentration of the macromolecule at the membrane surface and replaces the term C_G.

This equation can be rearranged to give the concentration of macromolecule at the membrane surface in terms of flux rate (Flaschel and Wandrey, 1979).

$$C_m = C_b . \exp \left(\frac{J}{K}\right) \qquad (4)$$

where :-

K mass transfer coefficient

In the case of a separation process C_b is the concentration in the feed stream.

The concentration C_m does not give any indication of the extent or concentration of the gel layer if formed. If the catalytic performance of the reactor is to be assessed the distribution of enzyme between soluble and gel entrapped states must be assessed.

Nakao et al., (1979) found that the resistance to flow of a gel layer

could be related to its concentration by an empirical equation of the form :-

$$R_g = A.C_g^B \qquad (5)$$

where :-

R_g resistance of the gel layer

C_g gel concentration

They found that B was a constant, independent of the polymer used. A was found to be characteristic of the polymer under test. As their experiments were carried out at constant pressure the validity of this equation for use in constant flux systems needs to be studied.

The effect of flux rates on transmembrane pressure drop were studied at a range of protein concentrations. The results were corrected for the effects of membrane resistance using the equation :-

$$\Delta p = J.(R_m + R_g) \qquad (6)$$

where :-

R_m resistance of the membrane

Δp transmembrane pressure drop

The experiments were carried out at 30°C using a volumetric recycle rate of 0.54 ml/sec (see Fig. 3.12).

Cursory examination of Fig. 3.12 shows a linear relationship between flux and pressure for low concentrations. Increasing the protein

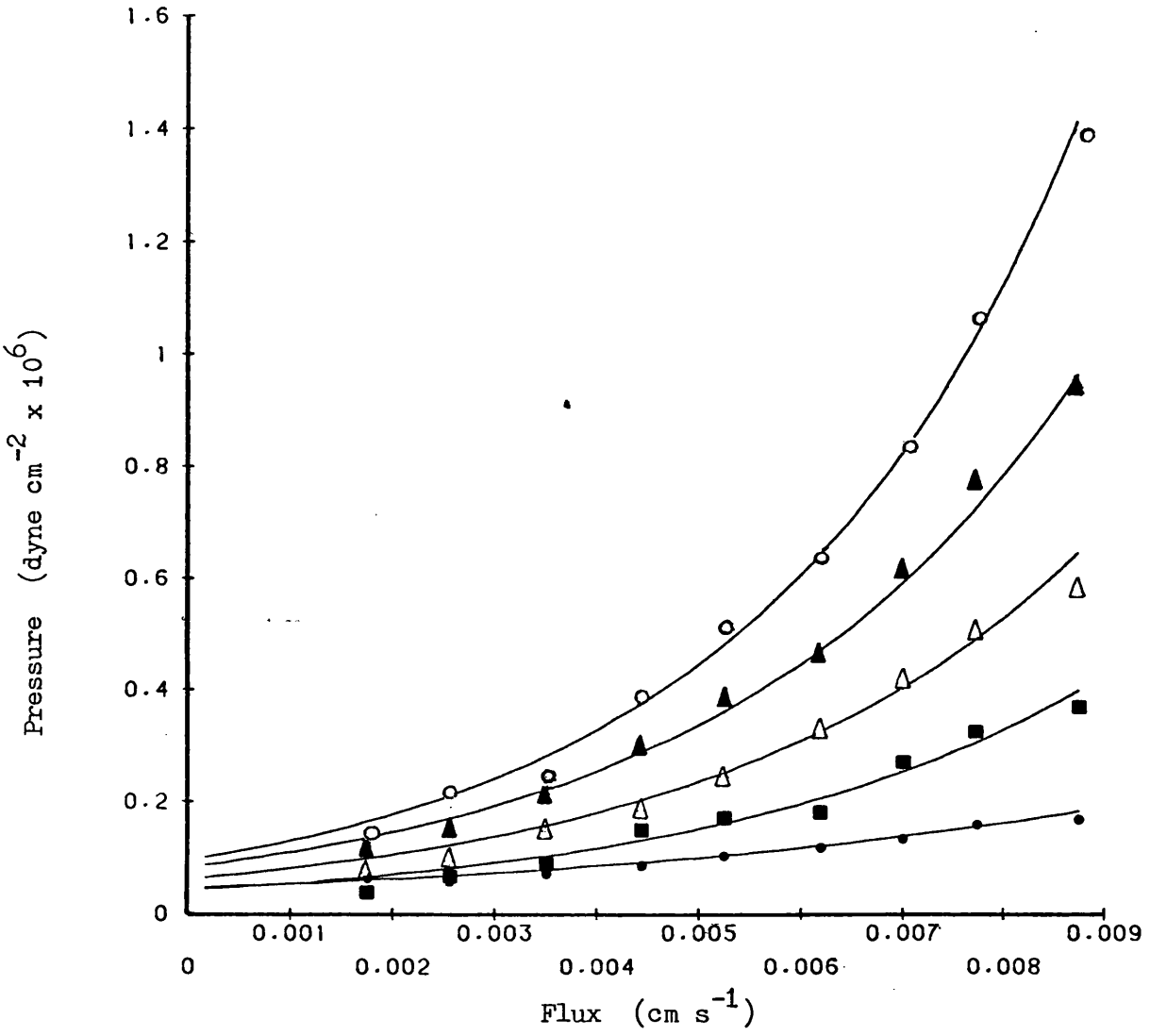


Fig. 3.12 Effect of Flux on Transmembrane Pressure Drop

(30 °C, volumetric recycle 0.54 ml sec^{-1})

- •—• 0.002% w/v BSA
- ■—■ 0.01 % w/v BSA
- Δ—Δ 0.02 % w/v BSA
- ▲—▲ 0.03 % w/v BSA
- o—o 0.04 % w/v BSA

concentration in the reactor causes an increasing deviation from linearity as would be expected from the exponential relationship expressed by equation 4.

To establish the applicability of the previously defined models, an attempt was made to correlate the transmembrane pressure drop with the protein concentration at the membrane surface. The first step in such an assessment is a calculation of the mass transfer coefficient. Equations relating mass transfer to wall shear and macromolecular diffusivity have been proposed for spiral thin channel systems in both laminar and turbulent flow regions. However, the use of pulsed flow and channels with acute bends prevents their direct use to describe this system.

Porter (1972) quotes typically observed protein gel concentrations of 25 - 45% weight to volume. If the assumption is made that at the maximum flux rate used, the membrane concentration is 45%, this value can be used to estimate the mass transfer coefficient of the system.

The bulk protein concentration at steady state can be calculated using a dynamic simulation of the reactor system. The protein concentration is described by two differential equations representing the net mass transfer in each direction :-

$$V_1 = J \times C_b \qquad (7)$$

$$V_2 = K \times C_m \qquad (8)$$

where :-

V_1 rate of polarisation

V_2 rate of back diffusion

$$\frac{dC}{dt}_b = V_2 - V_1 \qquad (9)$$

$$\frac{dC}{dt}_m = V_1 - V_2 \qquad (10)$$

These equations are integrated with respect to time for the initial conditions used. Using the results of the simulation and equation 4 the mass transfer coefficient can be solved by successive substitution. For the assumption of a maximum protein concentration of 45% (7×10^{-6} mole/cm^3) at the membrane the observed mass transfer coefficient is 9.3×10^{-4}.

This value was used to calculate the bulk protein concentration for each experimental point. The resultant membrane concentrations were calculated using equation 4.

The membrane concentration was calculated for all experimental values. If the resistance to flow is solely dependent on the macromolecule concentration at the membrane, it would be expected that this would be true irrespective of the conditions used. This was found not to be the case. Fig. 3.13 shows a plot of calculated C_m. The results suggest that pressure is a function of both C_m and flux. Figs. 3.13-14.

In view of the gel polarisation model this result can be interpreted as the formation of a gel layer, the gel concentration being flux

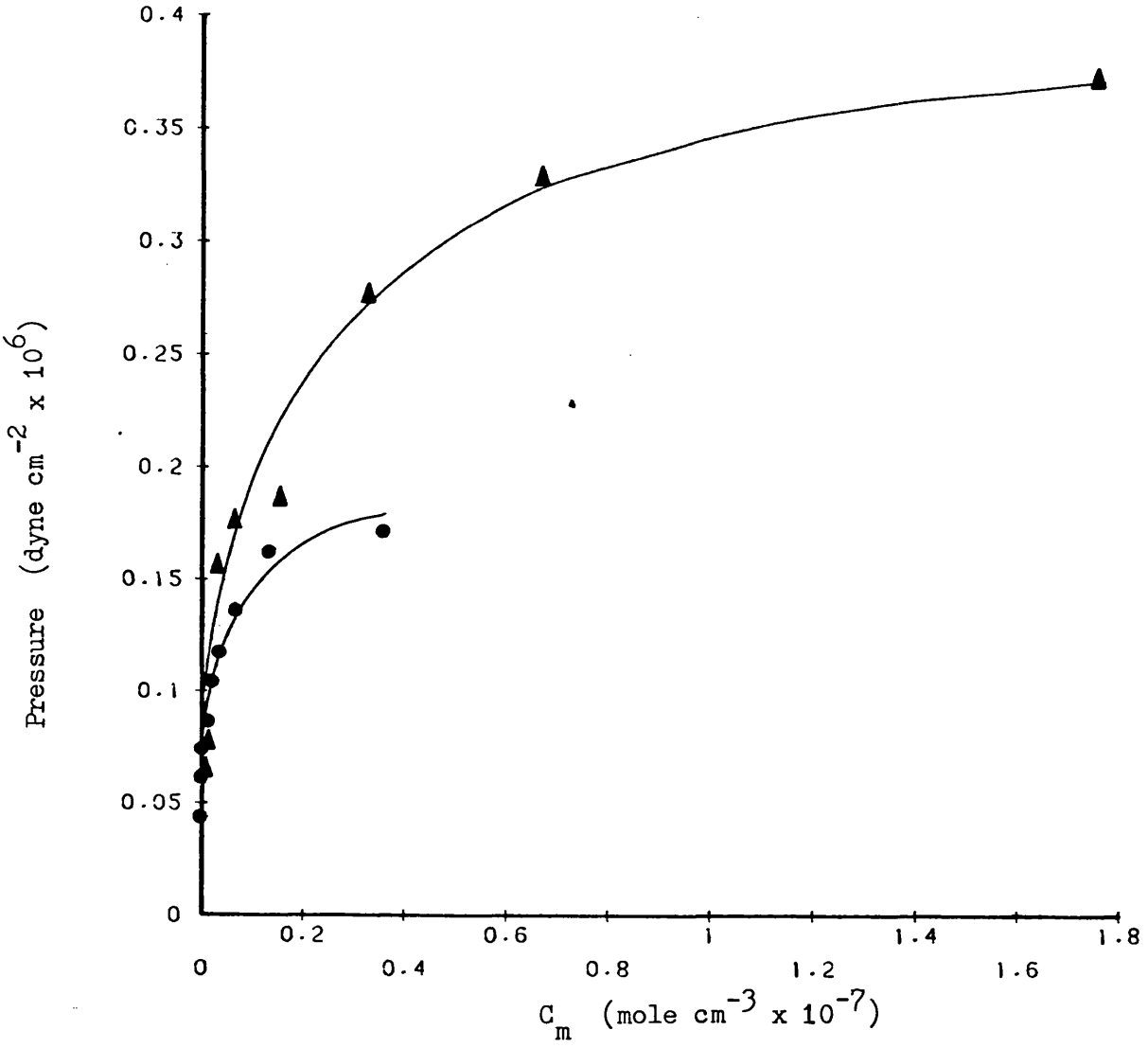


Fig. 3.13 Relationship between Calculated C_m and Transmembrane Pressure Drop

● —— ● Total loading 1 mg BSA

▲ —— ▲ Total loading 5 mg BSA

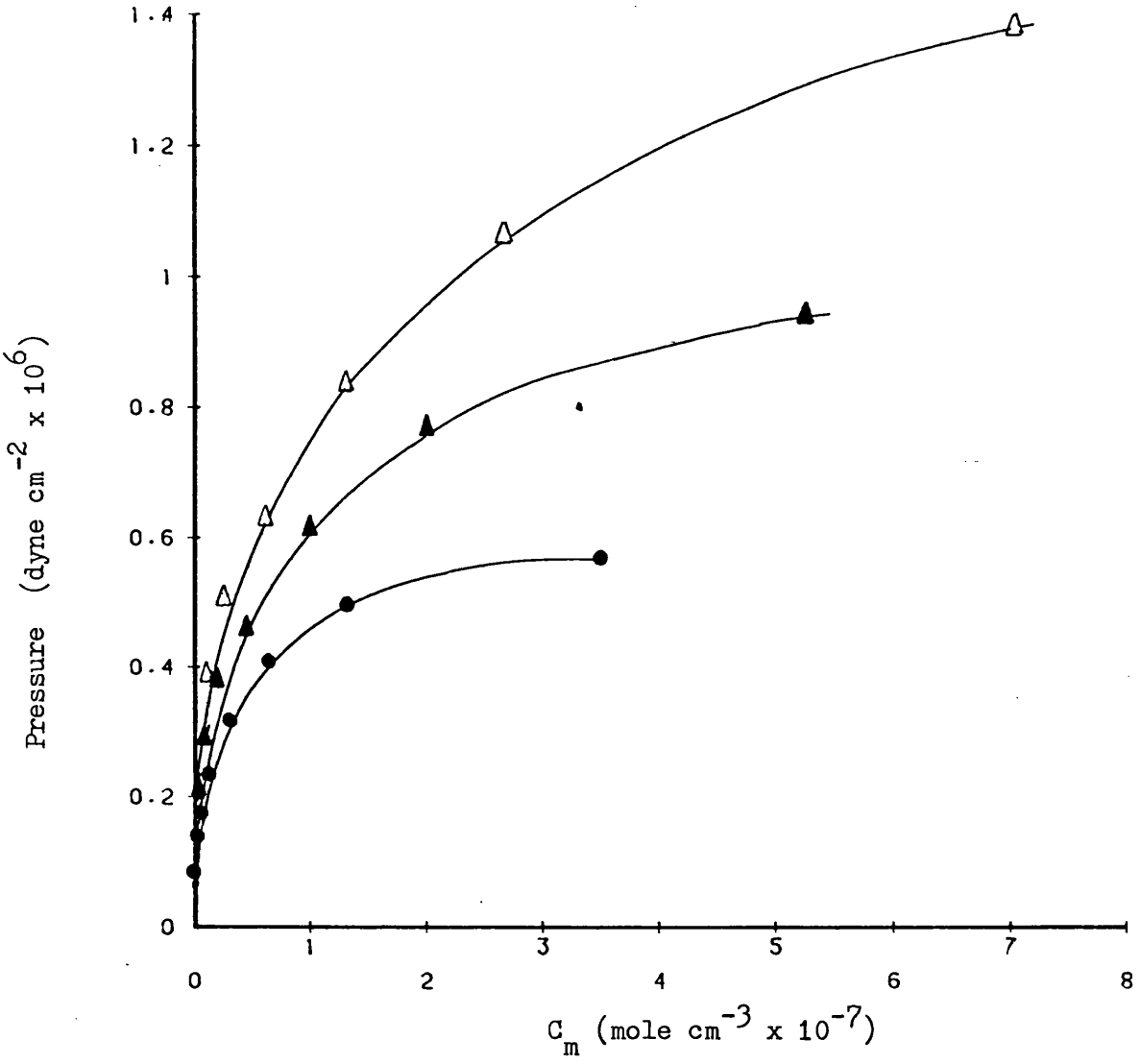


Fig. 3.14 Relationship between Calculated C_m and Transmembrane Pressure Drop

● —— ● 10 mg Total loading BSA

▲ —— ▲ 15 mg Total loading BSA

Δ —— Δ 20 mg Total loading BSA

dependent while the thickness is a function of the total protein present in the reactor.

To support this assessment the results were fitted to the equation proposed by Nakao et al., (1979) relating flow resistance to gel concentration.

Initial estimates of the parameters were made and the results were fitted using the non linear regression program proposed by Duggleby (1980).

The best estimates of the parameters are shown in Table 3.15

Table 3.15

Concentration mole cm^{-3}	A	B
3.125×10^{-10}	2.3	0.174
1.56×10^{-9}	2.9	0.282
3.125×10^{-9}	3.6	0.292
4.687×10^{-9}	4.8	0.305
6.25×10^{-9}	5.8	0.333

If the parameters A and B are plotted against the total weight of protein in the reactor (Fig. 3.16), it can be seen that A is linearly related to total protein while B appears to approach a maximum value. If A is considered as an empirical function of the gel layer thickness

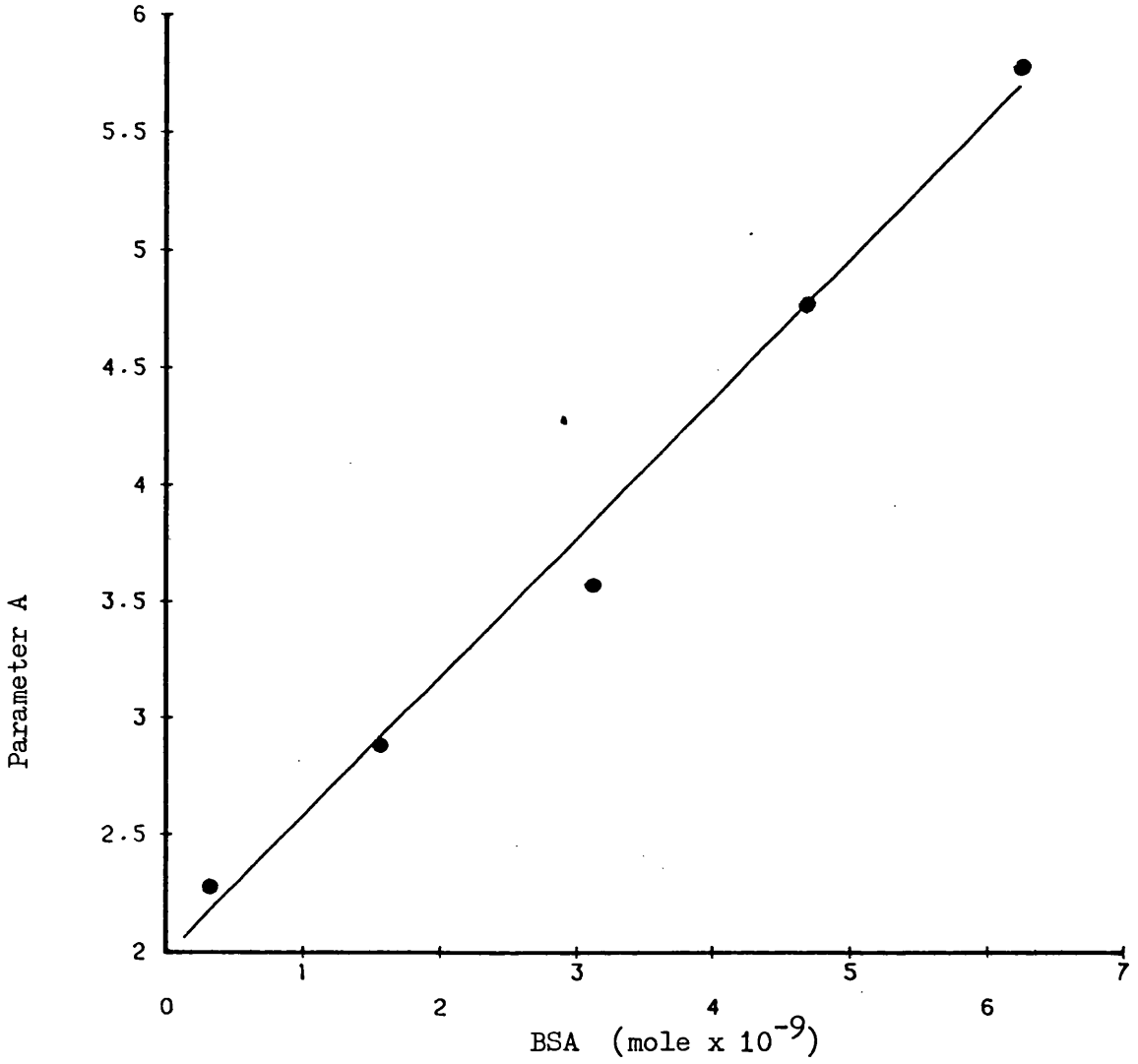


Fig. 3.16a Variation of Parameter A with Total Protein Loading

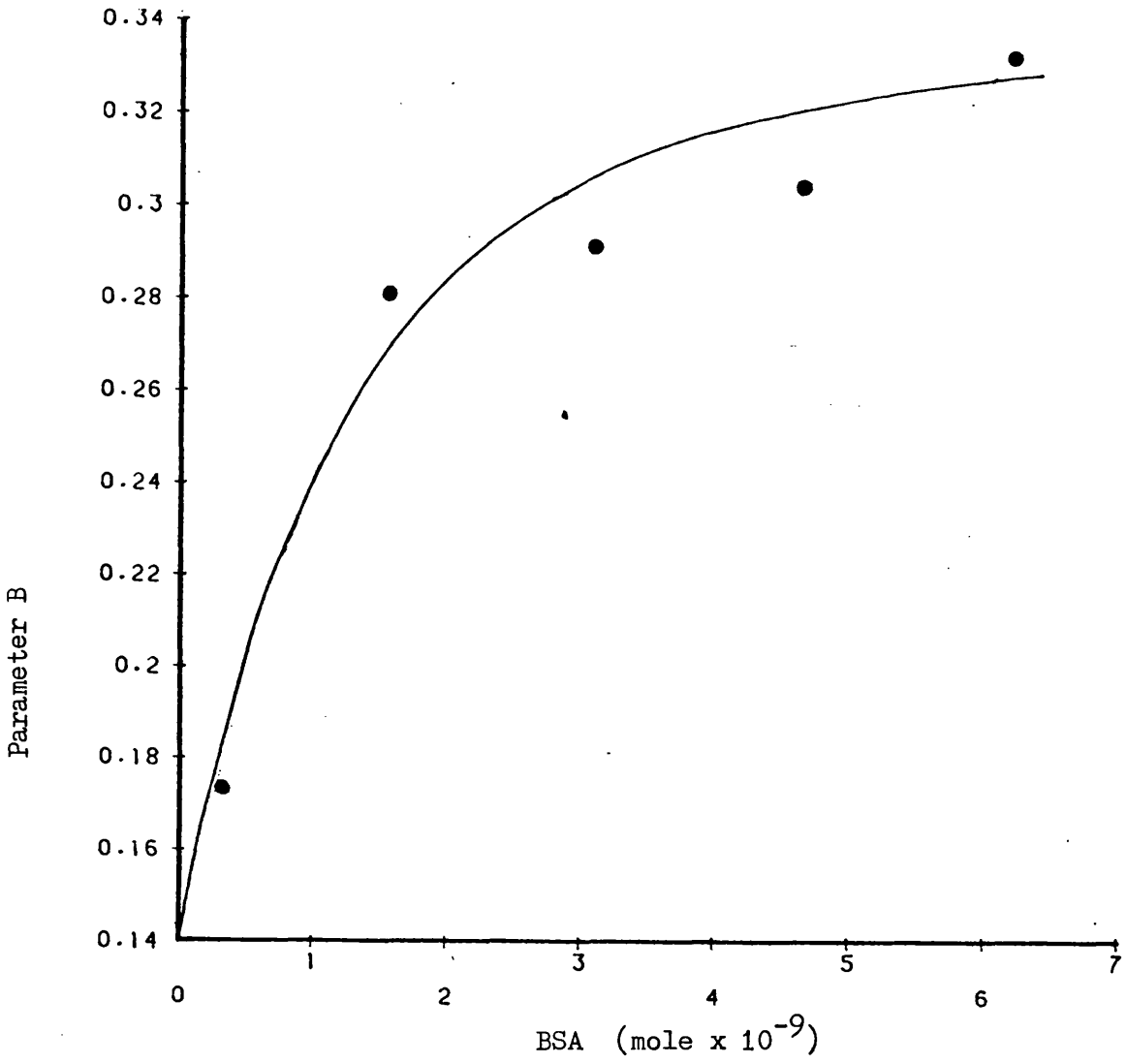


Fig. 3.16b Variation of Parameter B with Total Protein Loading

and B is considered as a function of concentration, the hypothesis is supported.

These results would appear to show a substantially different system from that described for conventional ultrafiltration systems where the gel layer is assumed to be constant. However, Porter (1972) points out the unexplained variation of gel concentration with reactor configuration. In their assessment of tubular membrane ultrafiltration systems, Nakao et al.,(1979) measured the gel formed on the membrane to test this assumption. They found a variation of concentration with the bulk concentration used, and the flux achieved. From their results they concluded that the constant gel assumption was invalid, a conclusion which supports the results reported here.

Effect of Volumetric Recycle Rate on Transmembrane Pressure Drop

The application of the conventially used mass transfer correlations is difficult in this case as the channel geometry and pulsed flow cause an increase in mass transfer in addition to the effects of the average recycle velocity.

From an examination of the channel geometry and the average velocity the Reynolds number can be calculated.

$$Re = \frac{u\ d\ p}{\nu} \qquad (11)$$

where :-

u	linear velocity
d	hydraulic mean diameter
p	density
ν	viscosity

The calculated Reynolds number can be used as a guide to the flow characteristics of the reactor. Work with thin channel systems has shown a transition from laminar to fully developed turbulent flow at Re = 2000. At the maximum recycle velocity used in this study the calculated Reynolds number was 270 based on hydraulic mean diameter, thus the reactor is operating in the laminar flow region.

The correlation used for mass transfer in laminar flow channels can be based on suitably modified solutions for convective heat transfer (Porter, 1972).

The general solution being :-

$$K = 0.816 \left(\frac{\gamma}{L} D^2\right)^{0.33} \qquad (12)$$

where :-

γ	fluid shear at the membrane surface
L	length of channel
D	diffusivity

Pulsed flow has been previously shown to decrease the effects of concentration polarisation (Gacesa, 1978. Grainger, 1973). This

can be considered as the effect of accelerating shear fields at the membrane surface. As the amplitude of the oscillation is a function of the volumetric recycle rate the most meaningful modification of the equation is the inclusion of a multiplying factor. Thus :-

$$K = 0.816 \left(\frac{d\gamma}{L}.D^2\right)^{0.33} \qquad (13)$$

where :-

d is a function of pulse amplitude and frequency

The effect of volumetric recycle rate on concentration polarisation was studied by repeating the previous experiments at lower volumetric recycle rates. Fig. 3.17 shows typical results obtained. The two rates used represented the maximum and minimum rates obtainable under the experimental conditions used, therefore a quantitative assessment could not be obtained.

To provide a situation where the effect of recycle rate was large enough to permit trends to be followed the experimental conditions were changed.

The reactor was loaded with 500 mg of Bovine Serum Albumin and was run with a linear flux of 1.75×10^{-3} cm s^{-1}. The effect of varying the volumetric recycle rate from 0.12 to 0.48 ml s^{-1} was studied at a temperature of 22 ^{o}C (Fig. 3.18). It was not possible to study a wide enough range of conditions to assess the power dependence of trans-membrane pressure drop on wall shear.

The results reported by Gacesa (1977) show that pulsed flow has a

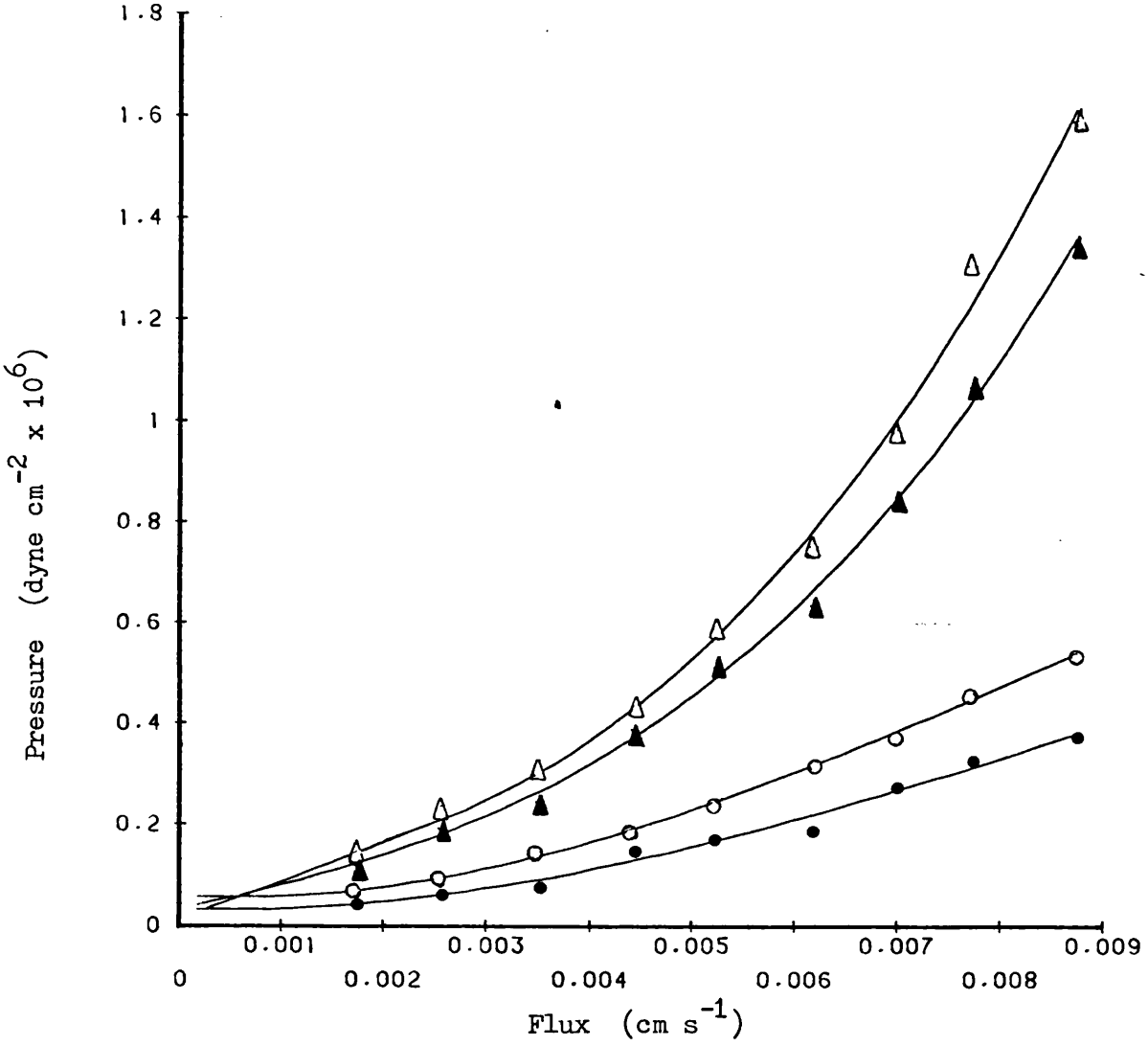


Fig. 3.17 Effect of Volumetric Recycle Rate on Transmembrane Pressure Drop

Total Loading 5 mg

●——● 0.54 ml s^{-1} recycle rate

o——o 0.18 ml s^{-1} recycle rate

Total Loading 20 mg

▲——▲ 0.54 ml s^{-1} recycle rate

Δ——Δ 0.18 ml s^{-1} recycle rate

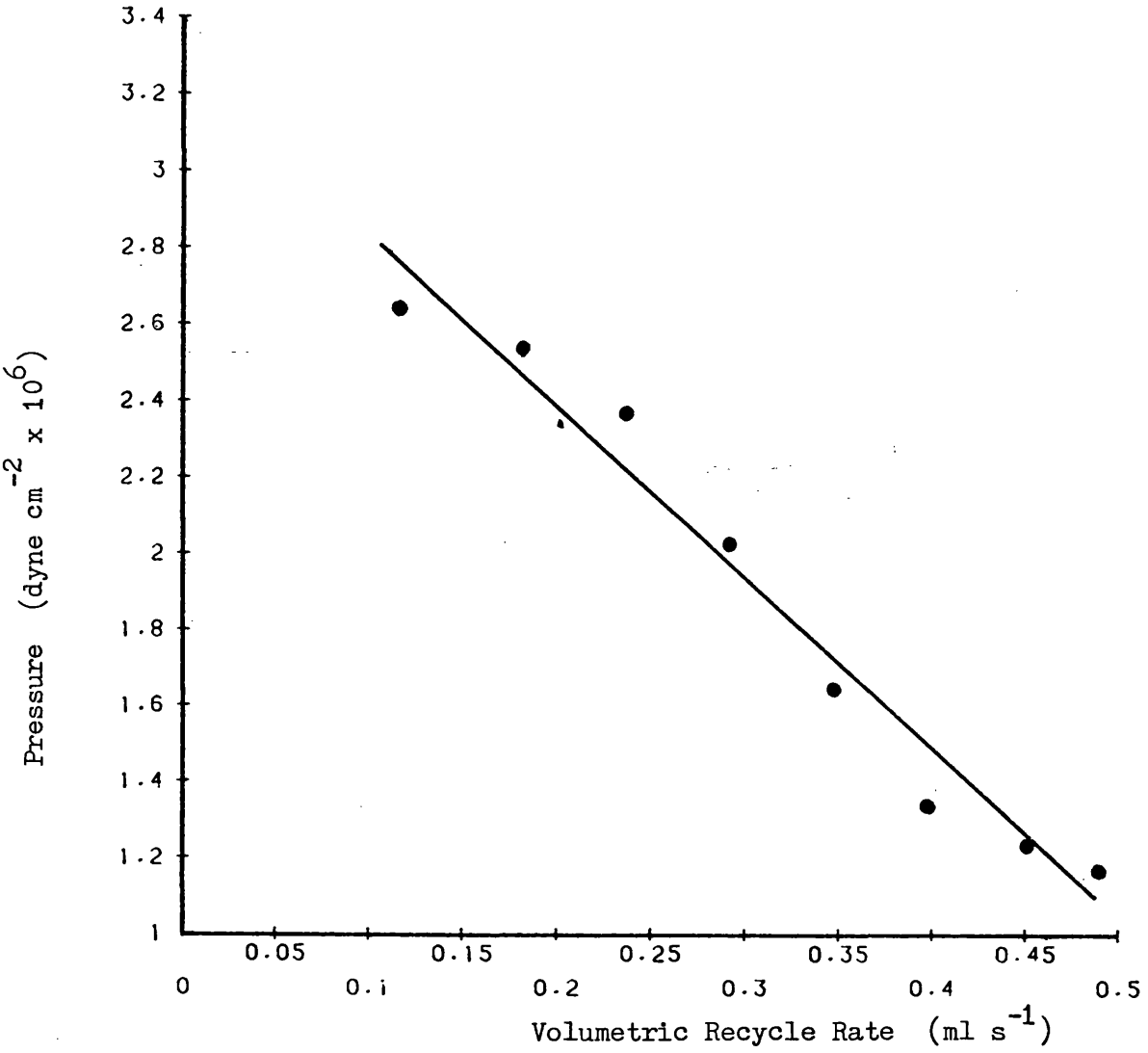


Fig. 3.18 Effect of Recycle Rate at High Protein Loading

greater effect than recycle rate over the range of conditions attainable in this reactor. This is seen in the discrepancy between the maximum predicted mass transfer coefficient and that estimated from experimental data (1.9×10^{-4} and 9.35×10^{-4}).

The Effect of Temperature

The relationship between temperature and mass transfer coefficient is described by the effect on the diffusivity of the macromolecule and on the viscosity of the solution.

The diffusivity of the molecule is described by the Stokes-Einstein relationship :-

$$D = \frac{kT}{6\pi \nu r_p} \qquad (14)$$

where :-

k	erg/deg	1.38×10^{-16} (Boltzman constant)
T	^{o}K	absolute temperature
ν	poise	viscosity
r_p	cm	radius of diffusing particle

As the bulk protein concentration is low the viscosity of the solution can be assumed to approach the value for water (Flaschel and Wandrey, 1979). The viscosity of water over the range 0-100 ^{o}C is reported in C.R.C. handbook of physics and chemistry (1974). Using these values and the Stokes-Einstein relationship the change in diffusivity can be calculated (Fig. 3.19). To study the effect of temperature on concentration polarisation a total of 30 mg protein was injected into

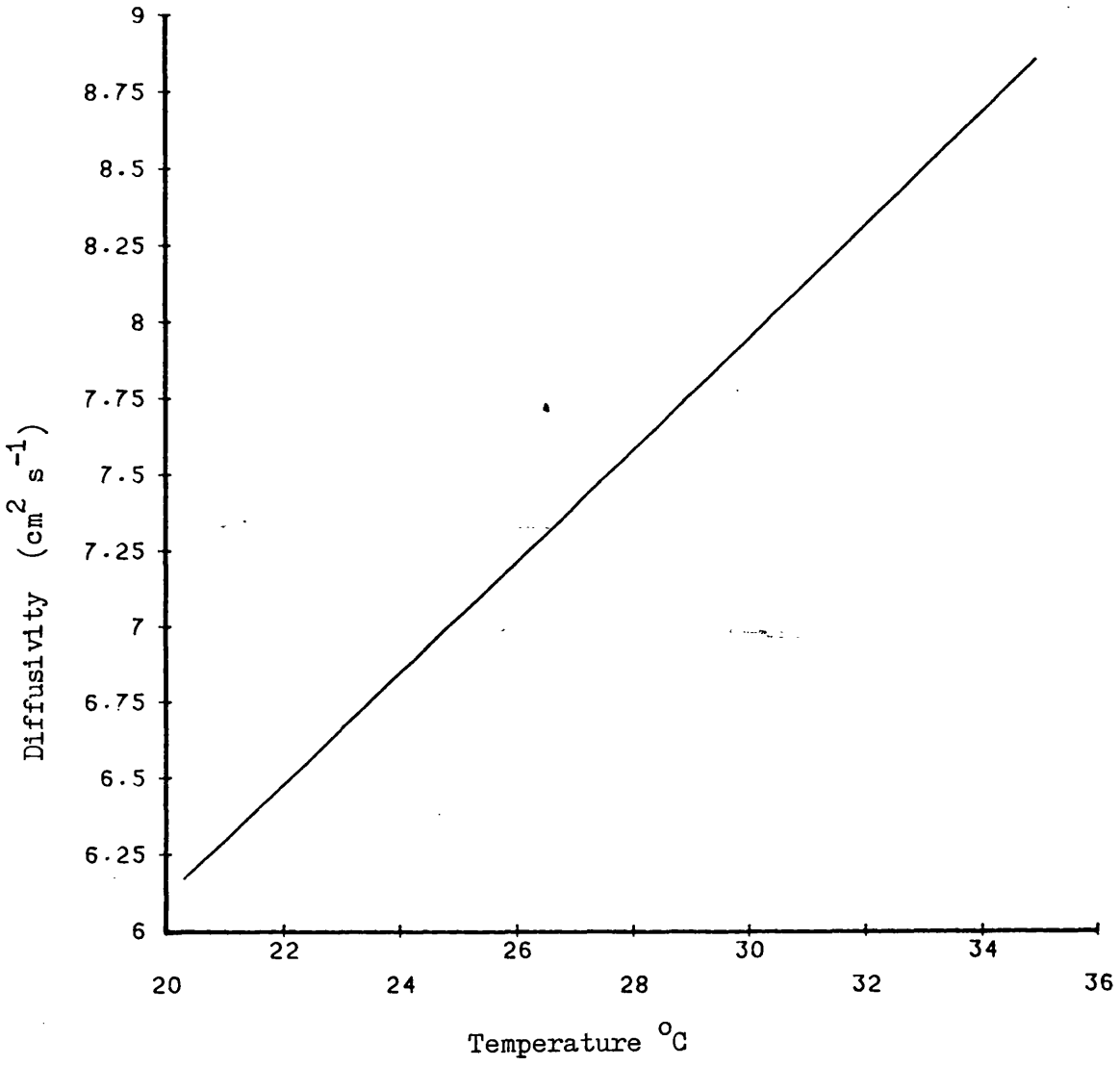


Fig. 3.19 Effect of Temperature on Diffusivity of B.S.A.

(calculated from equation 14)

the reactor. The transmembrane flux and volumetric recycle rate were set at 8.76×10^{-3} cm s^{-1} and 0.54 cm^3 s^{-1} respectively. The pressure was allowed to reach a steady state and then the effect of successive 1 °C step changes over the range 21 to 35 °C were studied (Fig. 3.20). The results show a linear decrease in pressure with increase in temperature over the range studied. This would be expected from the linear increases in diffusivity with temperature.

The effects of this increase in diffusivity on the dependence of pressure on flux and protein concentration can be seen in Fig. 3.21.

The Effect of pH and Ionic Strength

The effects of pH and salt concentration on concentration polarisation were studied by passing gradients through the reactor after it had reached steady state. The conditions were the same as the previous experiments (30 mg Bovine Serum Albumin, flux 8.76×10^{-3} cm s^{-1}, 0.54 cm^3 s^{-1} recycle) with the temperature kept constant at 21 °C. The effect of pH was studied from 4-9 and ionic strength over the range 10 mM to 1 M. No change in transmembrane pressure drop was observed in response to these changes. In view of the previous results, showing the effect of ionic strength on the attainment of steady state it would appear that the formation of the gel layer is influenced by these parameters but that once formed it is less affected with no changes being apparent over the time course of the experiment (3 hours).

In-Situ Membrane Regeneration

The cost of ultrafiltration membranes necessitates their reuse. On

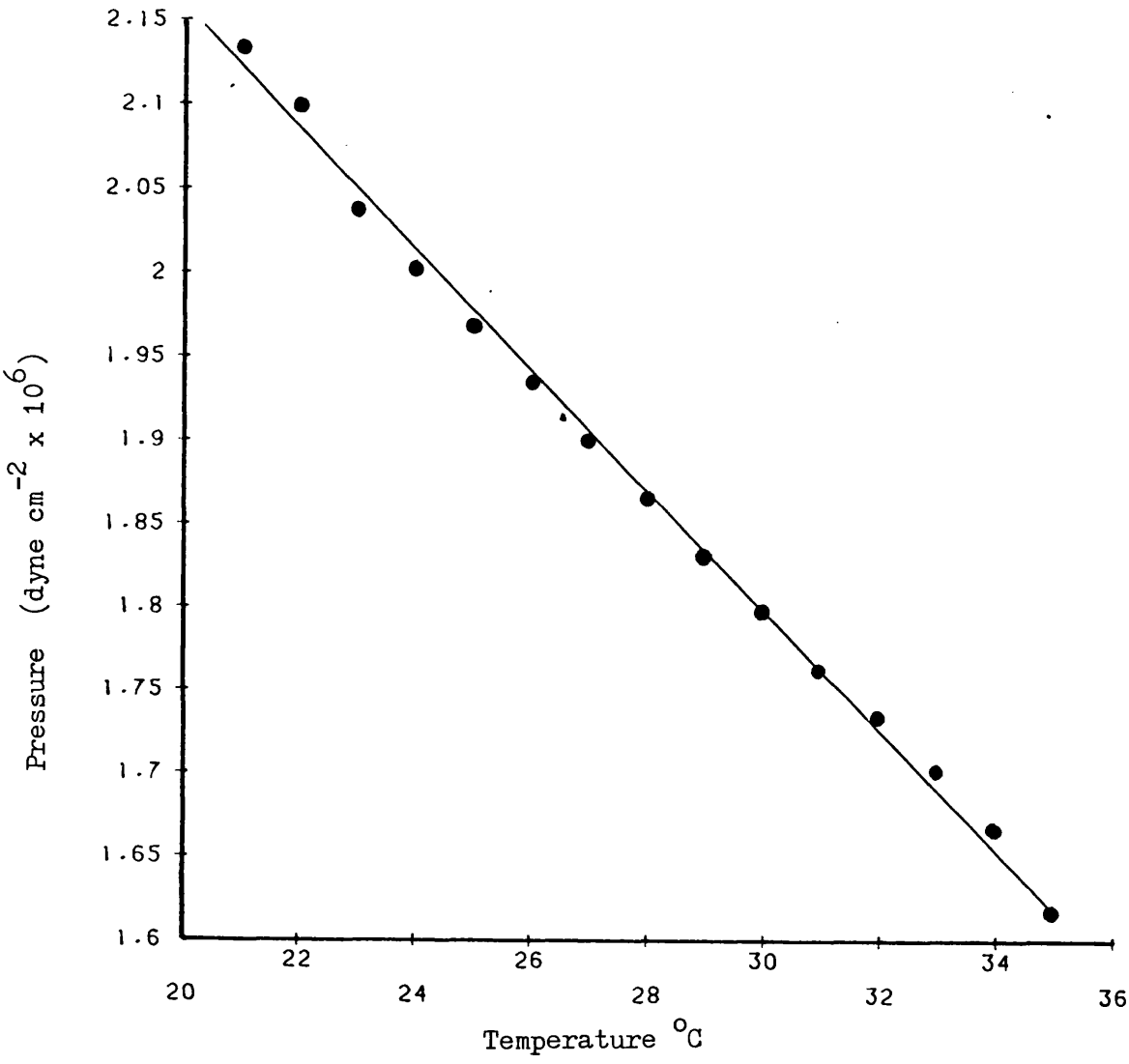


Fig. 3.20 Effect of Temperature on Transmembrane Pressure Drop

Recycle 54 cm^3 s^{-1}

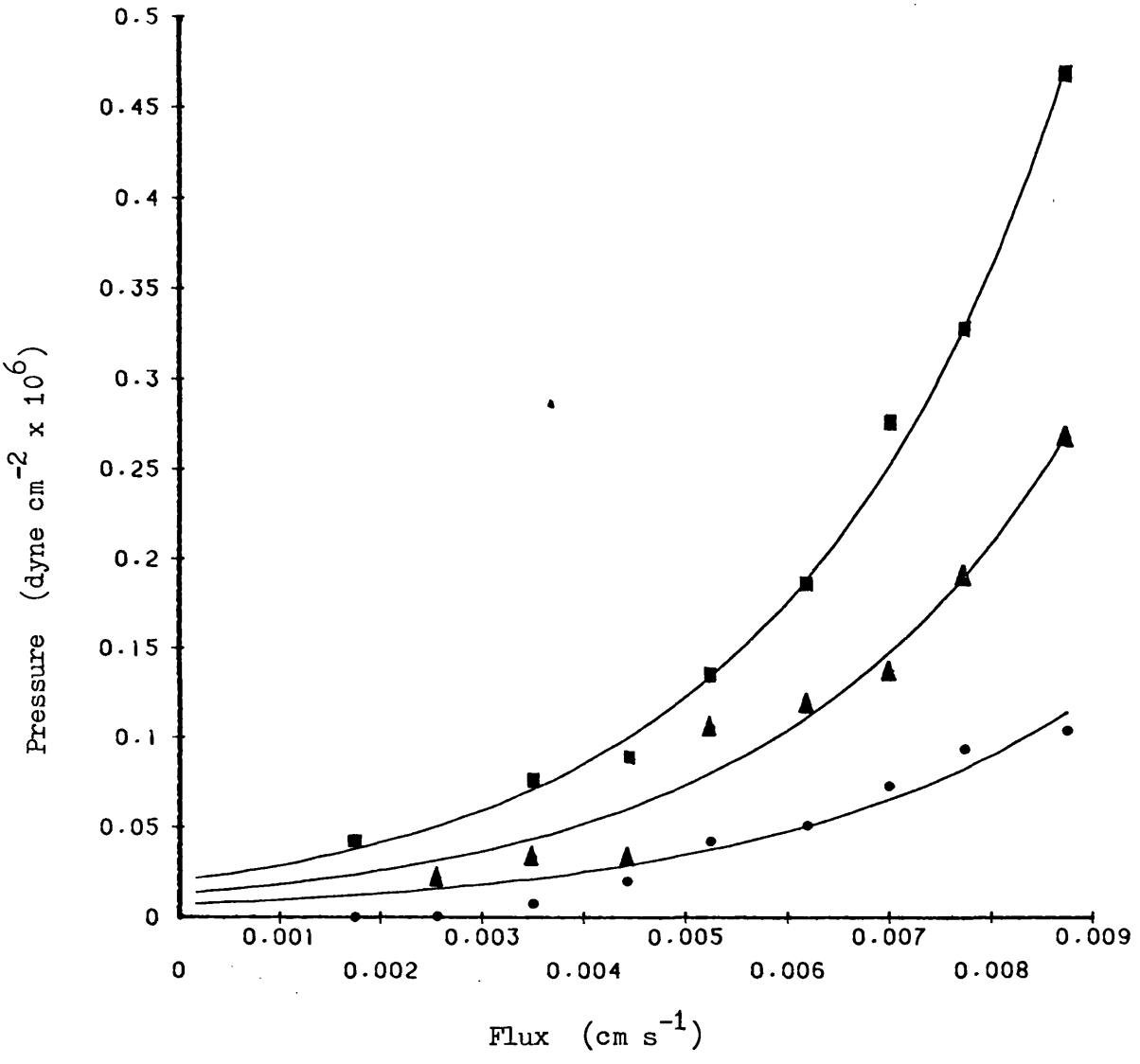


Fig. 3.21 Effect of Flux on Transmembrane Pressure Drop at 43 °C

●——● 10 mg Total Loading BSA

▲——▲ 20 mg Total Loading BSA

■——■ 40 mg Total Loading BSA

a laboratory scale it is simple to remove a membrane and remove gelled protein by washing or soaking in dilute acid or protease solution. For larger systems the use of detergents containing proteases have been suggested (Flaschel and Wandrey, 1979).

For systems requiring cofactors the advatages of using proteases are increased. Ideally the derivatised cofactors used in the reactor would have a greater stability than most enzymes currently available. Therefore it would be advantageous to remove denatured enzymes from the reactor without having to recharge with cofactor.

The effect of adding protease to the reactor was initially studied on a reactor containing 20 mg Bovine Serum Albumin. 1 mg Subtilisin was added and the reaction was allowed to proceed for 1 hour. Volumetric recycle rate was maintained at 0.54 ml/sec with zero transmembrane flux (Fig. 3.22).

This experiment was repeated using 40 mg Bovine Serum Albumin, the volumetric recycle rate was reduced to 0.19 ml/sec at a linear flux of 8.76×10^{-3} cm s^{-1} for the duration of the reaction (Fig. 3.23). Results obtained at the same flux with a recycle value of 0.54 ml/sec can be seen in Fig. 3.24.

Before the reactor can be reloaded with a fresh batch of enzyme the protease must first be denatured. Trichloroacetic acid solution at a concentration of 12.5% w/v is commonly used to denature enzymes and so the effect of subsequent addition of this solution to the reactor was studied. The pressure/flux relationships before and after treatment

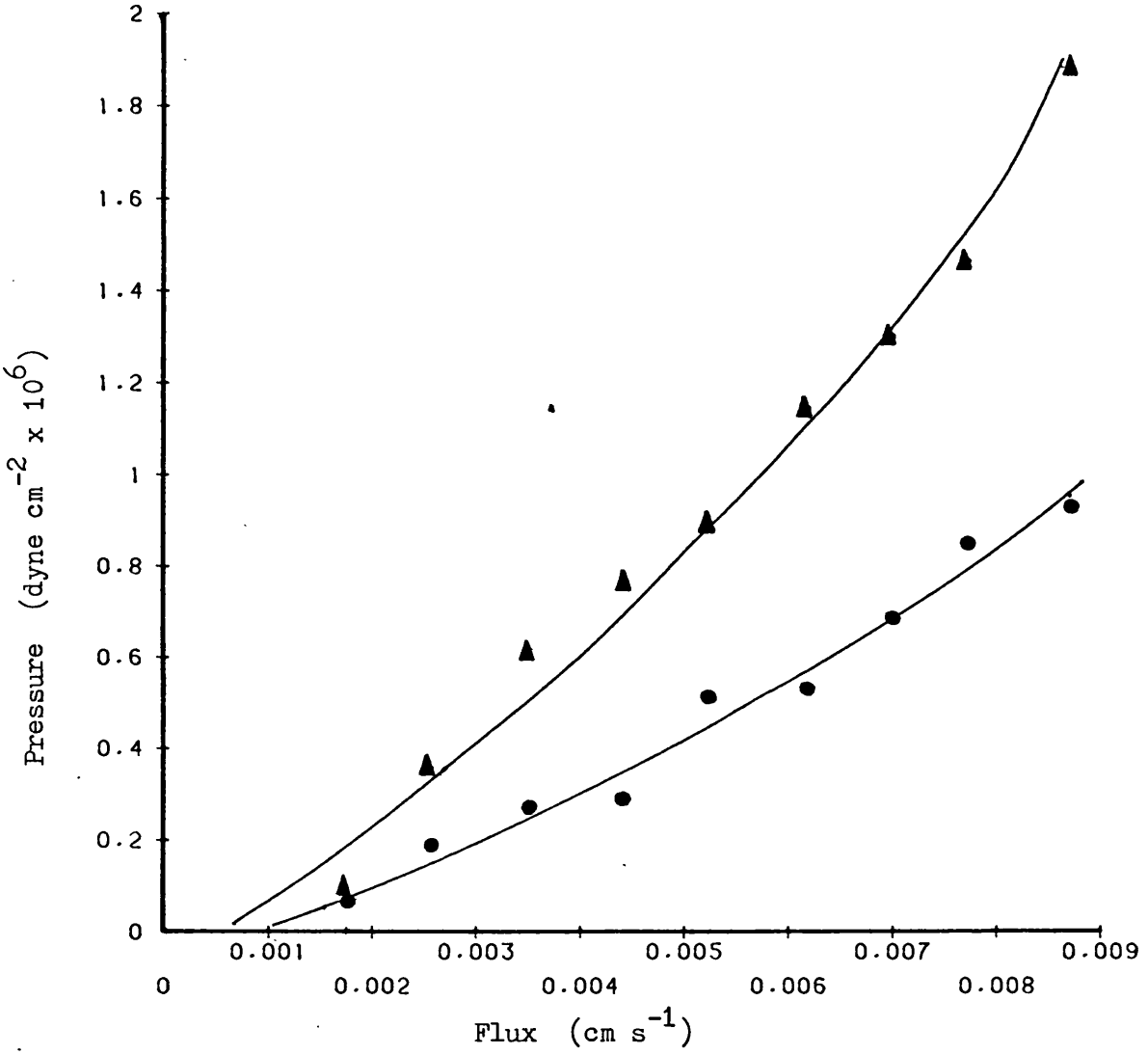


Fig 3.22 Effect of Protease Digest (approx. 20 °C)

▲——▲ Before incubation

●——● After incubation

(see text for details)

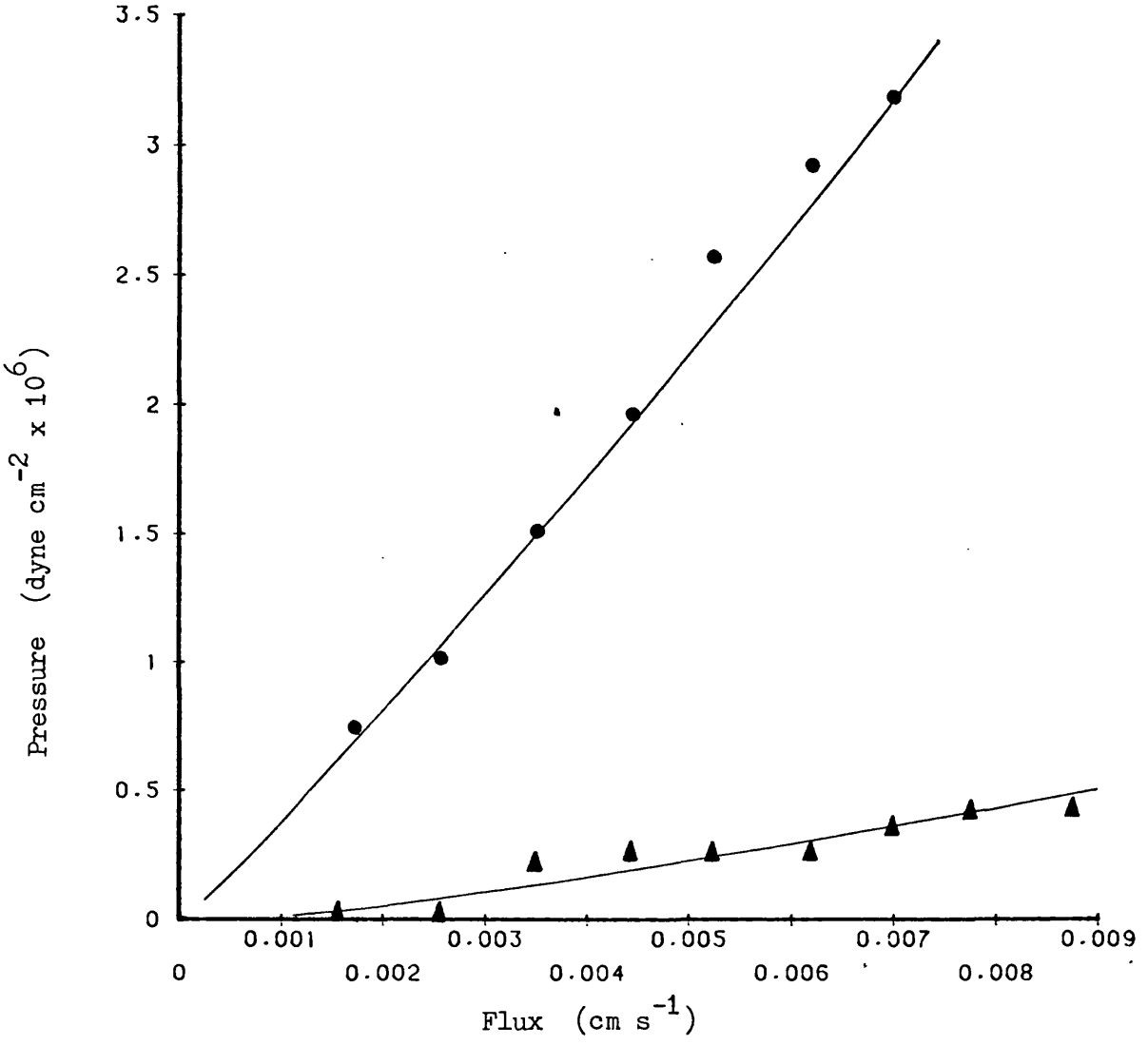


Fig. 3.23 Effect of Protease Digest (approx. 20 °C)

● —— ● Before incubation

▲ —— ▲ After incubation

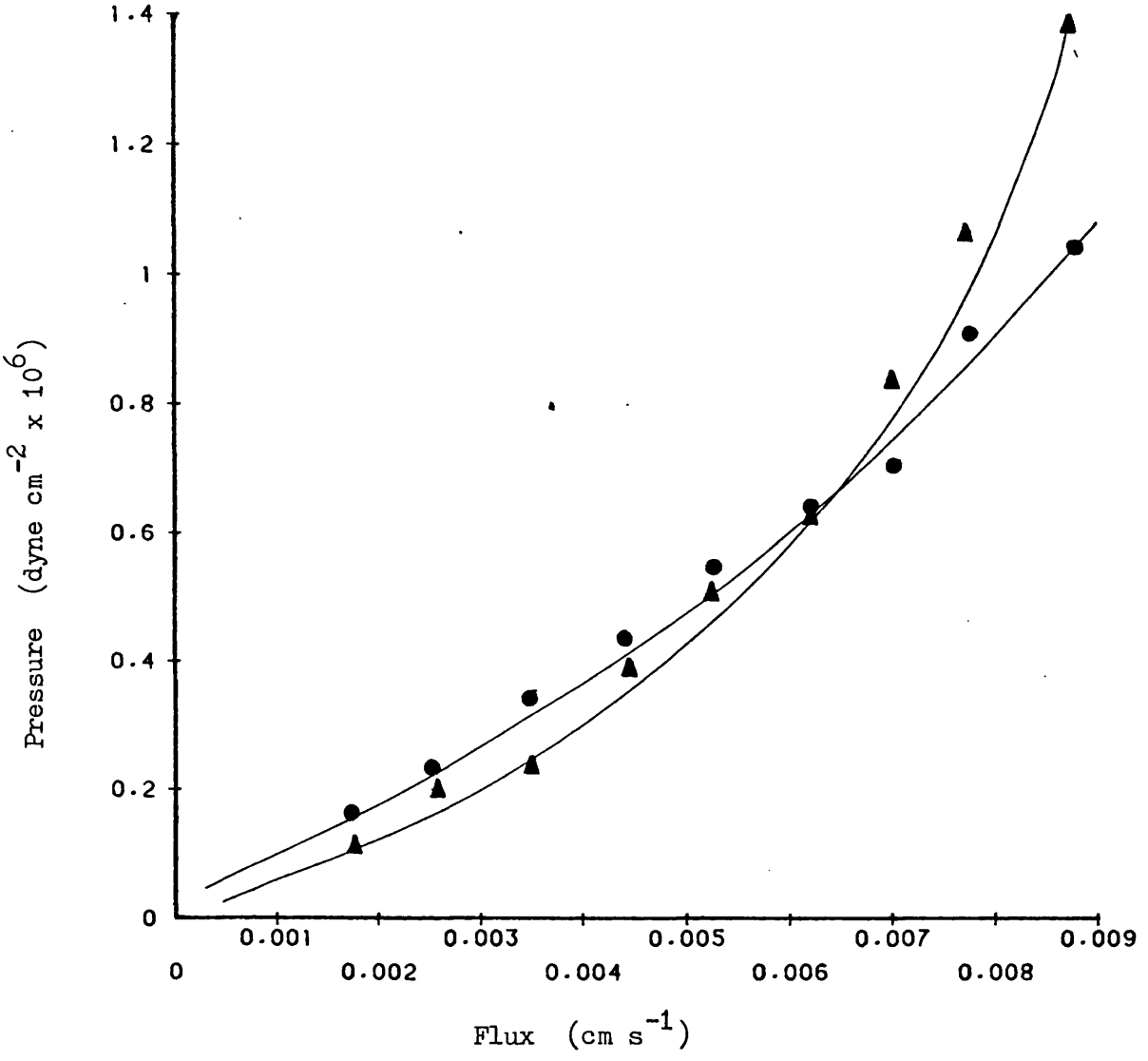


Fig. 3.24 Effect of Protease Digest (approx. 20 °C)

▲——▲ Before incubation

●——● After incubation

(see text for details)

with trichloroacetic acid can be seen in Fig. 3.25. This result shows that the precipitated protein is deposited on the membrane increasing the problems of concentration polarisation.

The results show that proteases can be used successfully in situ but further work is needed to study ways of destroying the residual protease activity without causing protein denaturation.

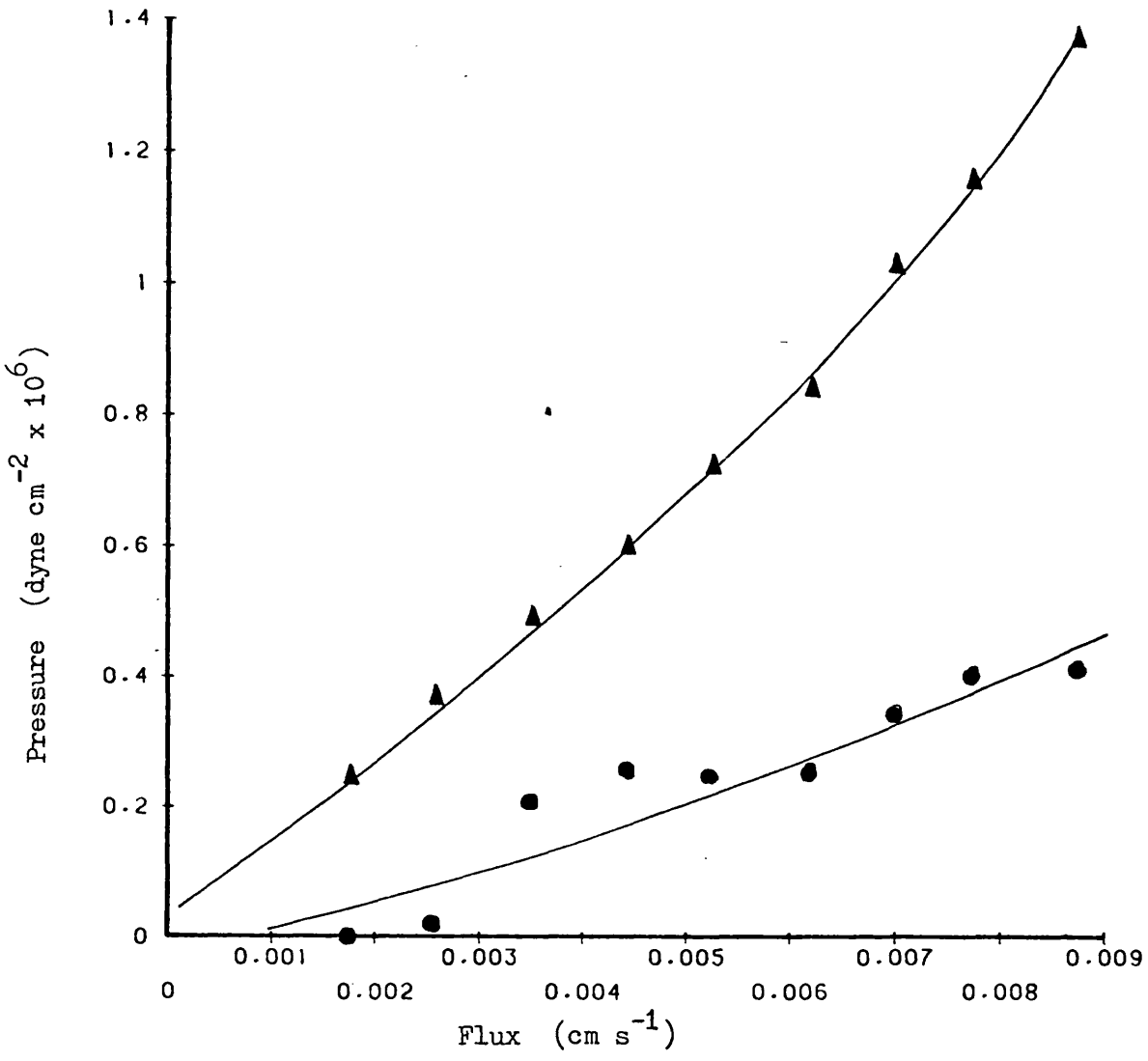


Fig. 3.25 Effect of T.C.A. Precipitation

▲——▲ After incubation

●——● Before incubation

(see text for details)

COFACTOR IMMOBILIZATION

The limited stability of the cyanogen bromide linkage for single point attachments suggested that this would not provide a solution to the problem of producing a high molecular weight cofactor analogue.

Ideally any such complex must be stable, simple and cheap to produce and active with a wide range of enzymes. An examination of the literature suggested that the method proposed by Sundberg and Porath (1974) would offer an ideal solution if modified for use with soluble dextrans.

The use of a bifunctional oxirane allows a simple two step coupling of a range of materials to sepharose gels. The resulting complexes were shown to be virtually completely stable for eight weeks at 70 ^{o}C in a solution of neutral or alkali pH.

Activation of Dextran

The method used was based on the method of Sundberg and Porath (1974). The reaction mixture was as follows :-

20 ml	0.25 M sodium hydroxide
40 mg	sodium borohydride
4 ml	1-4 - butane diol diglycidyl ether
	dextran

The sole variable was the amount of dextran used. In initial studies the dextran was dissolved in the sodium hydroxide/borohydride solution.

The reaction was started by slow addition of the diglycyl ether and allowed to proceed for eight hours with continuous stirring.

The maximum weight of dextran which could be used at this scale was 200 mg. Further increases in the amount of dextran led to the formation of a gel precipitate.

The activated dextran was separated by precipitation with approximately five volumes of cold ethanol. Recovery was by low speed centrifugation. The precipitated dextran was redissolved in distilled water and freeze dried.

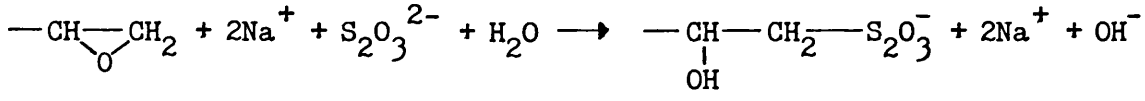
This freeze dried material was used for determining the number of oxirane groups present. The method used was proposed by Axen et al., (1975). Approximately 10 mg of complex was used in each case, the mixture was as follows :-

1 ml activated dextran solution

1 ml 2 M sodium thiosulphate

Each solution was adjusted to pH 7 before mixing and the reaction mixture was subsequently maintained at pH 7 by the addition of hydrochloric acid.

The reaction is as follows :-



The stochiometry is 1 mol OH^- per mol of activated groups.

The reaction was initially carried out using a Pye Unican pH meter with servo motors. This system had no proportional control facility and as a result large fluctuations in pH were observed throughout the course of the reaction.

The results obtained with 1-4 diglycyl ether were found to be far too high to be realistic allowing for the stoichiometry of 2 mol OH^- per mol of free diepoxide.

The pH fluctuations were considered to be responsible for these erroneous results and so the work was repeated using a Radiometer model PHM 26 pH stat.

The response of the instrument was first checked using a solution of tris buffer at known concentration. The method was then tested with free diepoxide, see Table 3.26.

The results obtained show that the epoxide sample used contained 85 % 1-4 diglycidyl ether by oxirane groups. This is consistent with the technical grade and age of the material. It is not possible to predict what percentage of molecules retain both oxirane rings; only that the figure lies between 65 - 85 %.

The demonstration of a viable assay for oxirane groups allowed the extent of dextran activation to be assessed. The effect of time on degree of substitution of sepharose gels had been studied by Sundberg and Porath (1974) but needed repeating for dextran.

Table 3.26 Determination of 1-4-Butanediol Diglycidyl Ether by Oxirane Titration

1-4-butanediol diglycidyl ether 25 mM	HCl 50 mM	% Oxirane
0.5 ml	0.425	85 %
0.5 ml	0.45	90 %
1 ml	0.85	85 %
1 ml	0.8	80 %
1 ml	0.85	85 %
2 ml	1.75	87.5 %

Average 85 % by active oxirane determination

The initial experiment studied the reaction over the time course of 1 - 6 hours. The reaction was set up as previously with aliquots being taken at the required time points, see Fig. 3.27. These results showed the maximum substitution to be reached within 60 minutes of incubation at ambient temperature (approximately 20 ^{o}C). Further incubation led to a decrease in the number of titratable groups. An explanation of this finding is that internal crosslinking occurs. This was substantiated by the decreasing solubility of samples incubated for the longer periods.

To study the early part of the reaction time course, the experiment was repeated with samples being taken every 10 minutes, see Fig. 3.28. The result confirms the reaction is essentially complete after 60 minutes.

The problem of decreasing solubility was considered to arise from two causes, firstly crosslinking occurring when the complex was precipitated with ethanol and secondly, crosslinking occurring during the incubation. To eliminate this problem, the effect of adjusting the pH to 7 before precipitating with ethanol was studied. The reaction was again found to be complete within 60 minutes, see Fig. 3.29. In this case no problem was found in redissolving the freeze dried material.

Binding of Cofactors

The initial method used was to continue from the activation step without precipitating the activated dextran. 250 mg ATP or ADP was dissolved in 8 ml 1.2 M sodium carbonate, and was added to the activation mixture and the pH adjusted to 9.5. The reaction was allowed to proceed for 24 hours with stirring. At this point glycine

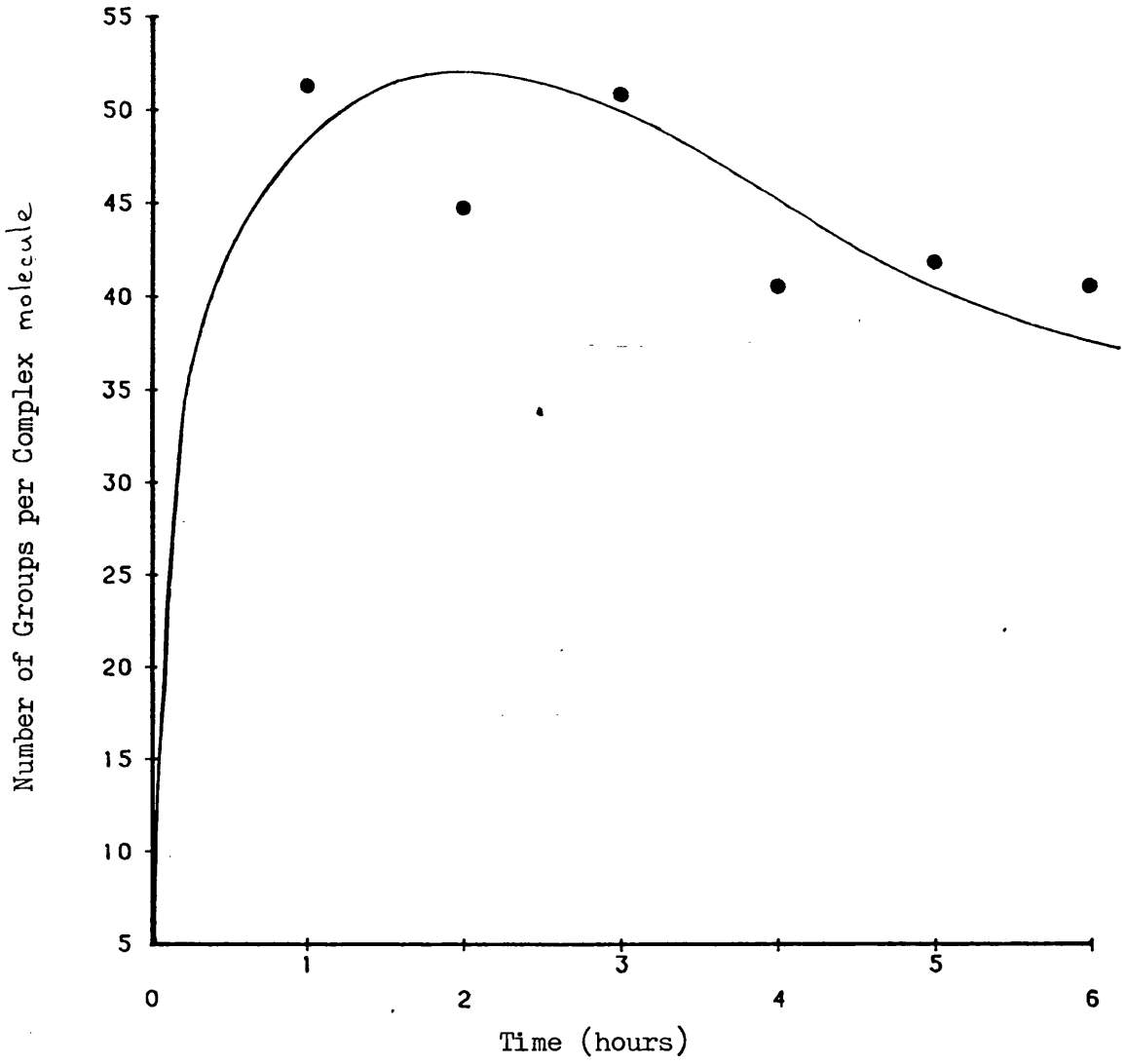


Fig. 3.27 Effect of Incubation Time on Dextran Activation

(all points are the average of three titrations)

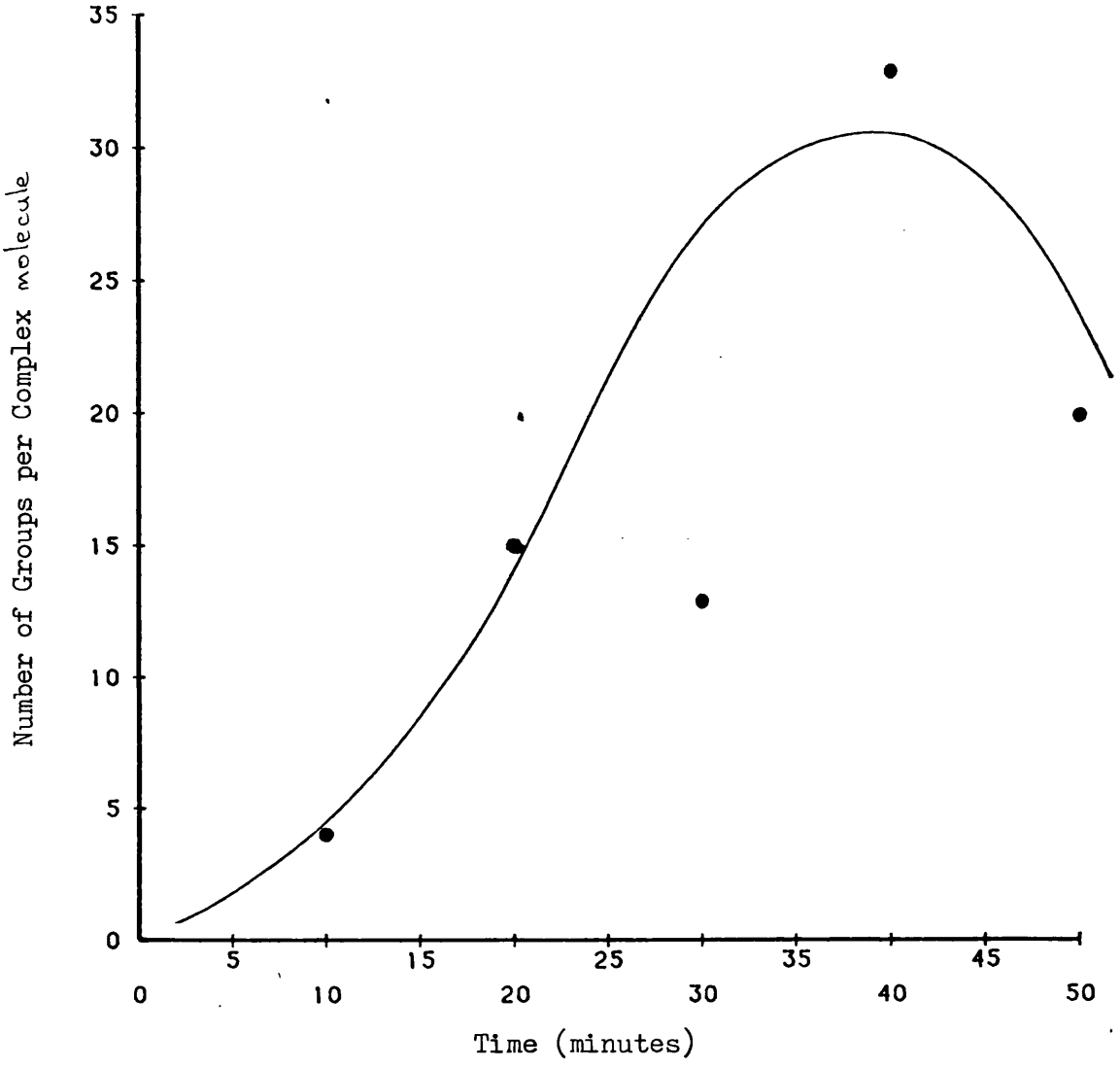


Fig. 3.28 Effect of Incubation Time on Dextran Activation

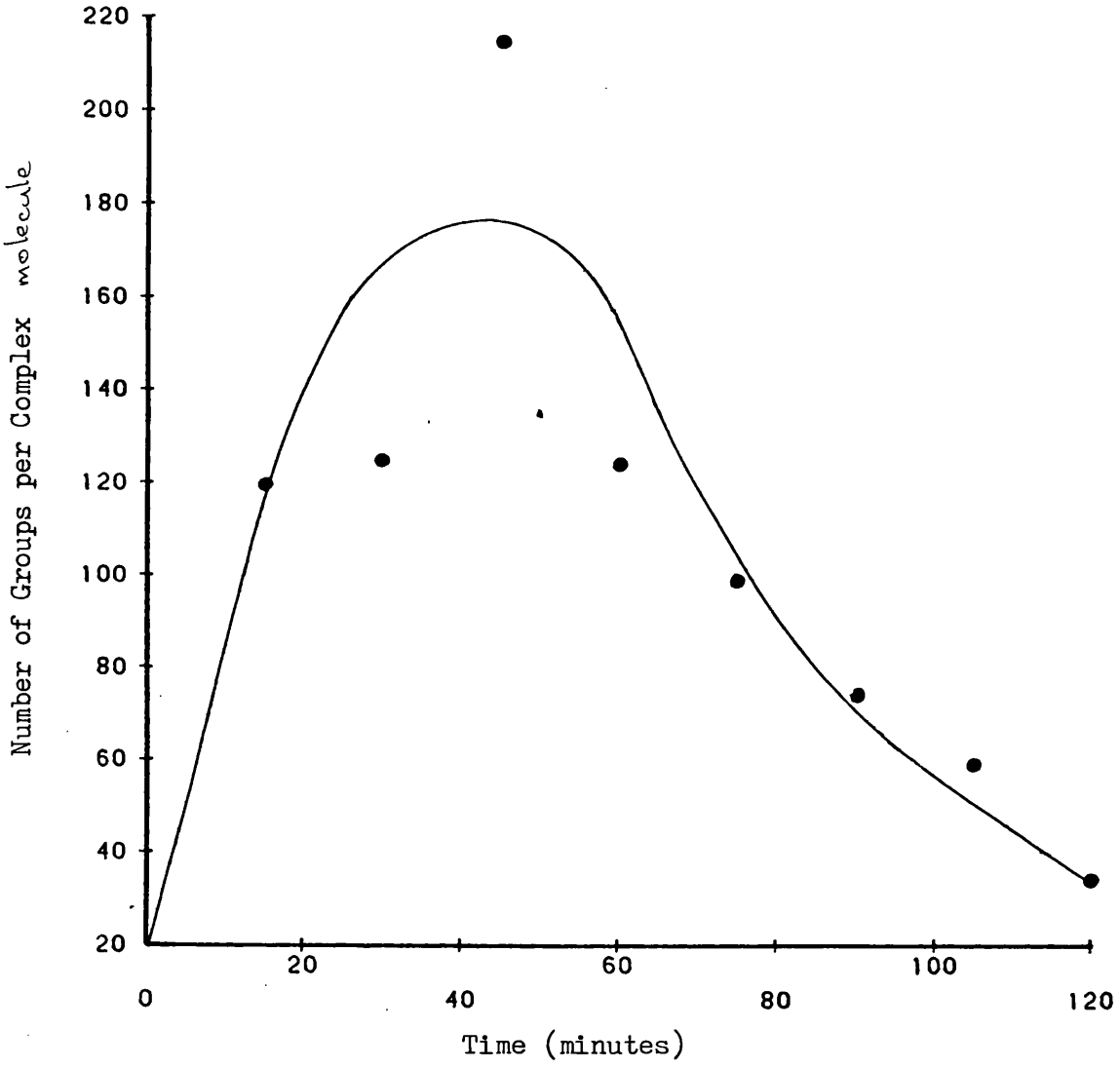


Fig. 3.29 Effect of Incubation Time on Dextran Activation

was added to block any remaining groups (final concentration 2 M). The complex was precipitated with 5 volumes of cold ethanol. The mixture was allowed to stand for 12 hours at -20 °C to allow maximum precipitation. Studies with pure dextran suggested a recovery of approximately 75% of the original dextran. This material was redissolved in a minimum volume of distilled water for purification studies.

Separation of Free from Bound Cofactor

Column Chromotography

Attempts were made to develop a simple one stage 'desalting' type separation using Sephadex G25 and Biogel P(2) columns run with 25 mM tris pH 7.5. These attempts were unsuccessful as the free cofactor came through in the void volume along with the cofactor dextran complex. Attempts to swamp any adsorption effects by running in 1 M salt did not improve resolution. The initial column size chosen was based on the manufacturers recommendation for fast desalting. Attempts to use larger columns improved resolution with Sephadex G25 giving a partial separation with a column of 40 cm x 0.6 cm. Biogel P(2) in a column of similar diameter offered no improvement. Although no obvious explanation could be made for these results, an attempt to separate a mixture of three dyes (Bromophenol Blue, Bromophenol Purple, Napthol Green) using Biogel P(2) showed that the elution volumes were not related to their formula weights. No further attempts were made to improve the separation as the size of column required was considered to be impractical for large quantities.

Affinity Chromotography

An alternative separation based on affinity of Concanavalin A for glucose was attempted. Concanavlin A was coupled to Sepharose 4B using the cyanogen bromide activation. The amount of protein bound was estimated by difference and was found to be approximately 8 mg/ml. The capacity of the resultant gel for dextran was measured as follows. A 5 molar excess of dextran was added to the affinity matrix and then stirred for 2 hours at room temperature in 25 mM tris, pH 7.5. The slurry was packed into a short column and sequentially eluted with 5 volumes of distilled water, molar acetic acid and 4 M acetic acid. The eluant from each wash was pooled and freeze dried. The dextran was found to be eluted in the 4M acid and the result was found to correspond to approximately 50% the capacity based on total protein bound i.e. 3.6 mg dextran per ml gel.

This experiment was repeated using the cofactor dextran complex but no specific elution could be achieved. The column was monitored at 254 nm while elution was carried out. The bulk of the material appeared to be washed out with water with neither 1M or 4 M acetic acid affecting the eluent. It is possible that the dextran is substituted to an extent that prevents binding to the immobilized Concanavlin A.

Dialysis

Dialysis was considered to be the simplest technique remaining to be tried. It has been previously used by Fuller and Bright (1977) for the purification of ATP substituted polyacrylamides. The primary

disadvantage of dialysis is the time required for separation; in addition large volumes of buffers required. It has the advantage of being an inexpensive separation technique.

The standard ATP-dextran preparation was dissolved in 10 ml of water and dialysed against water for varying periods. The amount of unbound cofactor was estimated by measuring the retention in an ultrafiltration cell using an Amicon PM 10 membrane.

The results showed that dialysis offered a simple, effective method of removing unbound cofactor and was ideal for use in preparing laboratory quantities.

Ultrafiltration

Ultrafiltration offers several advantages over dialysis as a method of separation. Several commercially available 'dialysers' comprise hollow fibre ultrafiltration membranes which allow materials to be dialysed and then concentrated very rapidly. The major advantage of ultrafiltration is that the unbound cofactor can be recovered without the excessive dilution necessary for dialysis. This offers the potential of reuse in larger scale productions.

The system used was a stirred tank with a 90 mm Amicon PM 10 membrane. The nominal tank volume was 210 ml. The reactor was charged with unpurified dextran-ATP. Distilled water was pumped through the reactor with stirring, the effluent stream being continuously monitored for absorbance at 254 nm. The optical density of the effluent returned to

zero after the passage of approximately 5 residence times. No attempt was made to optimise the flow rate but flow would be constrained by concentration polarisation.

The use of stirred tank demonstrated the feasibility of using ultrafiltration as a separation process. An interesting possibility arising out of this result was the potential to use the reactor to purify the dextran-ATP before injecting the enzymes.

Molecular Weight Determination

Having demonstrated the successful coupling of cofactors to dextran T40 some attempts were made to characterise the molecular weight of the resulting complex.

Gel Filtration

The elution of dextran-ATP was compared with the elution of various dextrans from a Sepharose 4B column. The dextran standards were labelled with red procion dyes. The dye (5 mg) was allowed to react with a solution of 400 mg of dextran in 1 ml, giving a complex with measurable absorbance at 254 nm. The column of dimensions 85 x 1.5 cm was shown to have a void volume of 43 ml using carbon particles. All sample bands applied to the column showed severe broadening which may reflect the range of molecular weights present in the dextran preparation. The elution pattern from the column was the inverse of the expected result with the smallest standard being eluted first and the largest last. The elution volumes obtained are shown in Table 3.30

Table 3.30

Eluent	Elution Volume
Carbon particles	43 ml
T40-ATP	149 ml
T40	175 ml
T70	183 ml
T500	204 ml

All elutions were carried out at a flow rate of 10 ml/hour in molar sodium chloride. The results suggest some retardation effect possibly a charge effect which invalidates any conclusions of molecular weight

Viscometry

Macromolecules cause large increases in the viscosity when in solution. This arises from distortions in the flow pattern of the liquid and is a function of the molecular weight and the shape of the macromolecule.

The intrinsic viscosity of a macromolecule can be related to molecular weight by the empirical Mark-Houwink equation :-

$$(\eta) = KM^{\alpha} \qquad (1)$$

where :-

K and α constants for the system

The intrinsic viscosity can be calculated from the specific viscosity by the empirically derived Huggins equation :-

$$\frac{\eta_{sp}}{C} = (\eta) + K'(R)^2.C \qquad (2)$$

where :-

η_{sp}		specific viscosity $\frac{(\text{(soln)} - \text{(solv)})}{\text{(solv)}}$
(η)		intrinsic viscosity
C	%(w/v)	concentration
K'		Huggins constant

Thus a plot of η_{sp}/C versus C will have an intercept ( ) and a slope of $K'(R)^2$.

Experimental values were obtained using a suspended level viscometer in a temperature controlled water bath. The specific viscosity was calculated from the relative flow times of solvent and solution. Results are shown in Fig. 3.31.

The Huggins constants were calculated from equation (2) and were found to be 0.45 for dextran T40 and 0.6 for dextran based ATP. Work by Senti et al., (1955) had shown a variation in K' of between 0.44 and 0.57 for dextrans of the range 18,000 to 100,000 daltons. This allowed the calculation of the constants in the Mark-Houwink equation :-

$$(\eta) = 1 \times 10^{-3}.M_v^{0.5} \quad \text{at } 25\ ^{o}C$$

Using these values and experimental results obtained the molecular weights were estimated as 33,000 for dextran T40 and 80,000 for dextran-ATP.

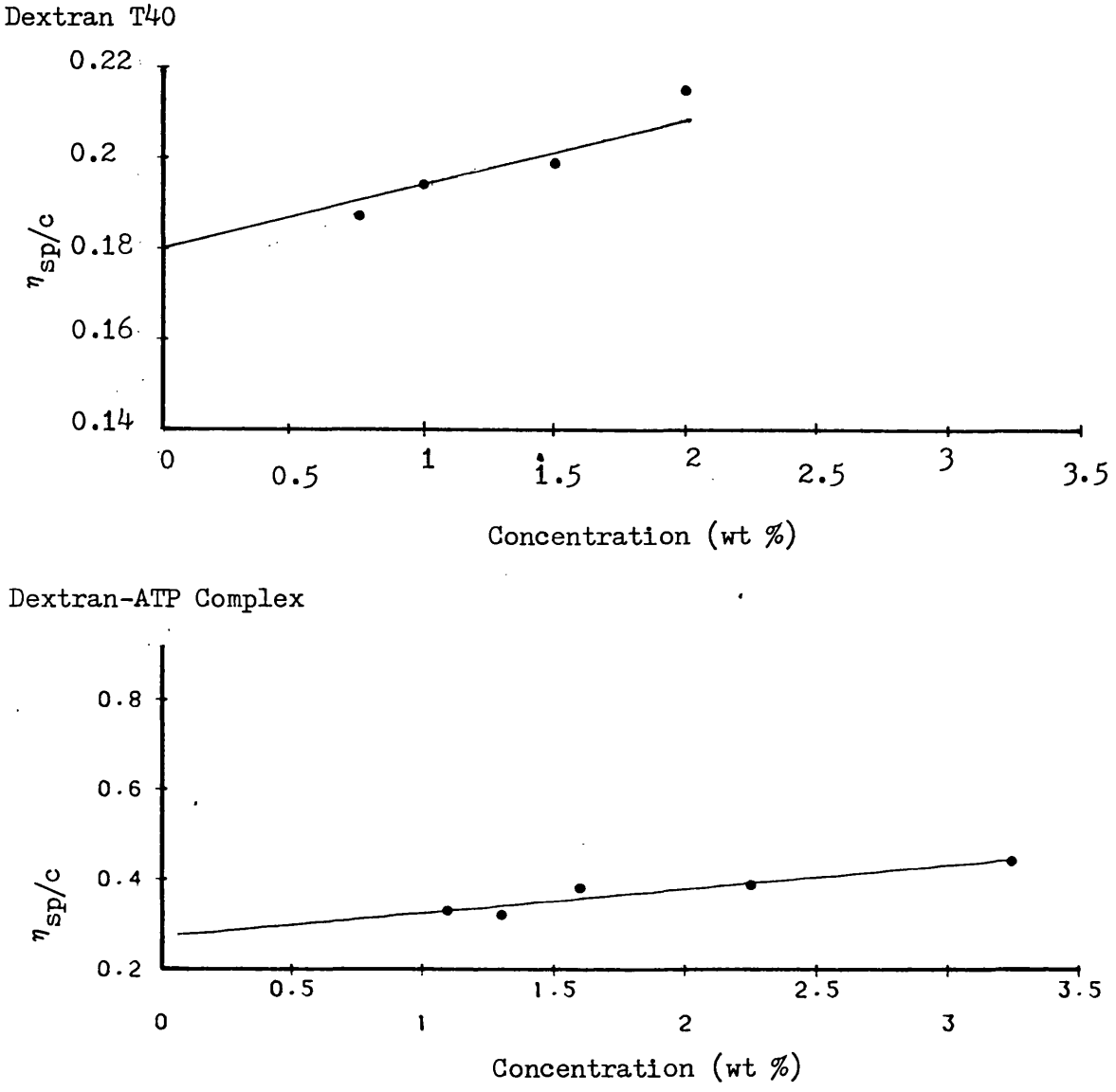


Fig. 3.31 Viscosity of Dextran and Dextran-ATP

(Viscosity was measured against 0.9% saline at 25 °C. all measurements were in triplicate. Lines were fitted using a linear regression program.)

Characterisation

Initially all the results were calculated for a known freeze dried weight of complex. As it is time consuming and inconvenient to dialyse and freeze dry samples before measurement, methods of determining the dextran content of the complex in solution were studied.

Colourimetric Dextran Assay

The dextrans used in this study are polymers of glucose. Thus the ends of the dextran chains possess reducing groups which can be detected using conventional colourimetric assays. The Nelson test for reducing sugars produced a linear curve for glucose and free dextran, see Fig. 3.32. The results for molar equivalents of both components indicate an average of one reducing end per dextran molecule. Attempts to repeat the assay using ATP-dextran proved unsuccessful and were invalidated by the formation of an insoluble complex in some instances.

Estimation by Refraction

The results obtained from affinity chromotography and the Nelson test suggest that chemical methods do not offer a reliable means of estimating the dextran content of the complex.

An alternative approach is to make a standard preparation of complex which can be used as a reference in a physical quantification. In earlier preparations any unreacted oxirane groups were blocked by coupling to glycine. As ATP has been shown to bind to approximately

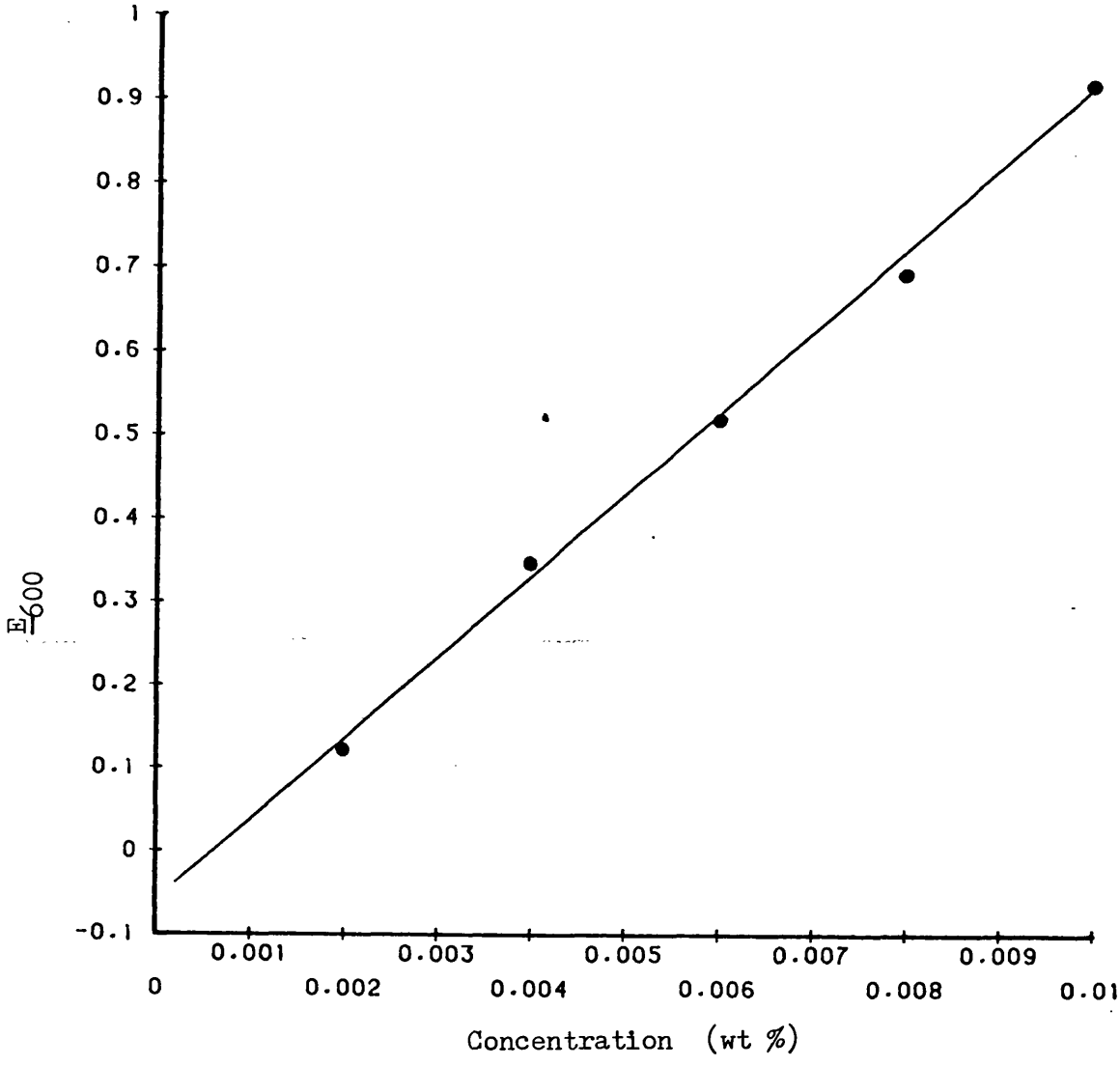


Fig. 3.32a Standard Curve for Glucose

(Results represent the average of duplicate values.

Lines fitted using a linear regression program.)

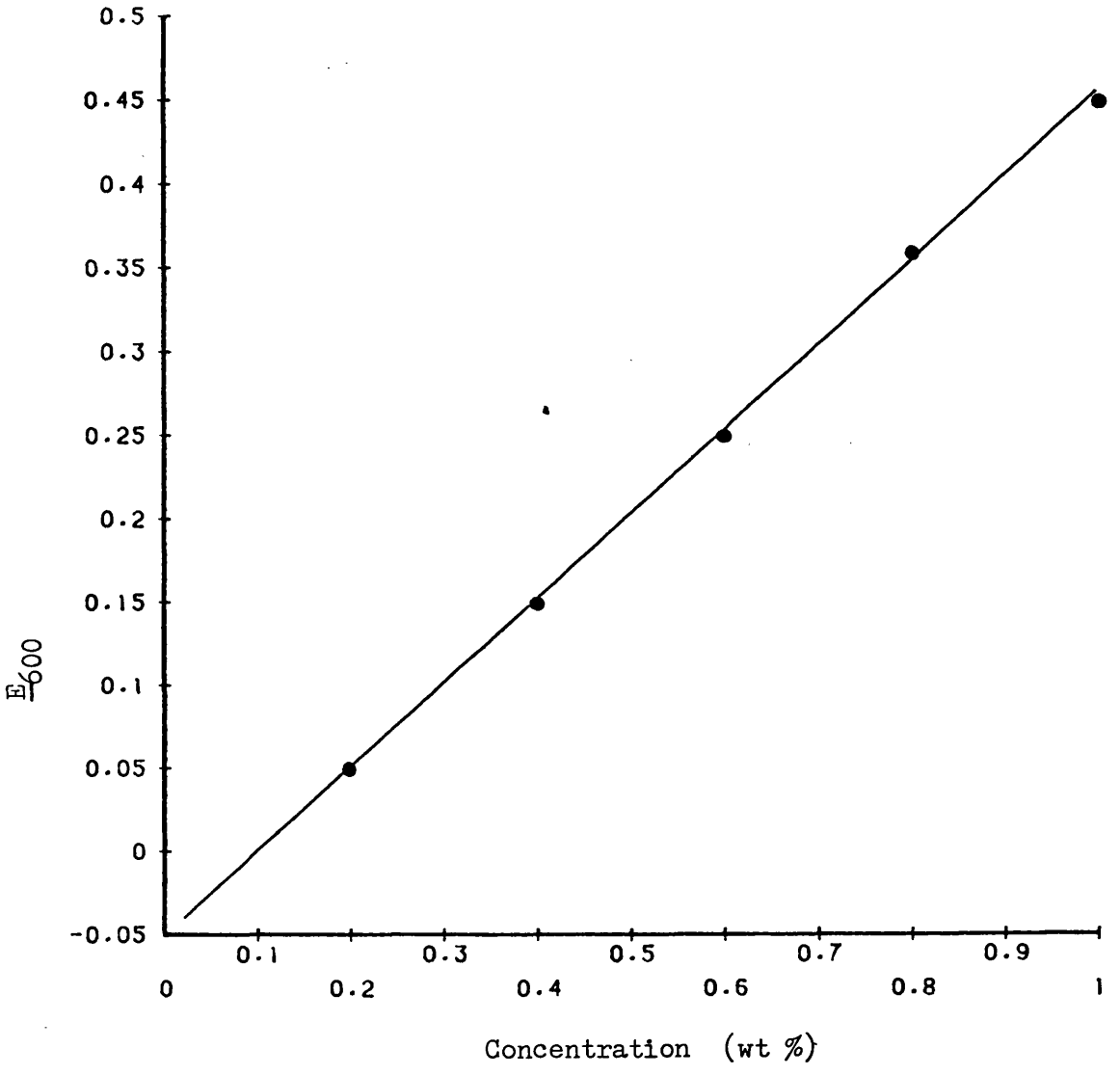


Fig. 3.32b Standard Curve for Dextran T40

10% of the available groups the standard preparation would be physically similar to a complex comprising solely dextran-glycine.

A convenient parameter to measure is the refractive index. This parameter is a function of temperature and density of the solution being measured. The specific refractive power can be expressed :-

$$S = \frac{n^2 - 1}{n^2 + 2} \cdot \frac{1}{d} \qquad (3)$$

where :-

S	specific refractive power
n	refractive index
d	density

This equation can be multiplied by the molecular weight to give the Molecular Refractivity :-

$$R = \frac{n^2 - 1}{n^2 + 2} \cdot \frac{M}{d} \qquad (4)$$

where :-

M	molecular weight
R	molecular refractivity

R is a function of the substance used. This offers the potential to calculate the molecular weight if dextran and ATP-dextran complexes show similar R values.. This was found not to be the case. Fig. 3.33 shows the divergence obtained even at low concentrations. However,

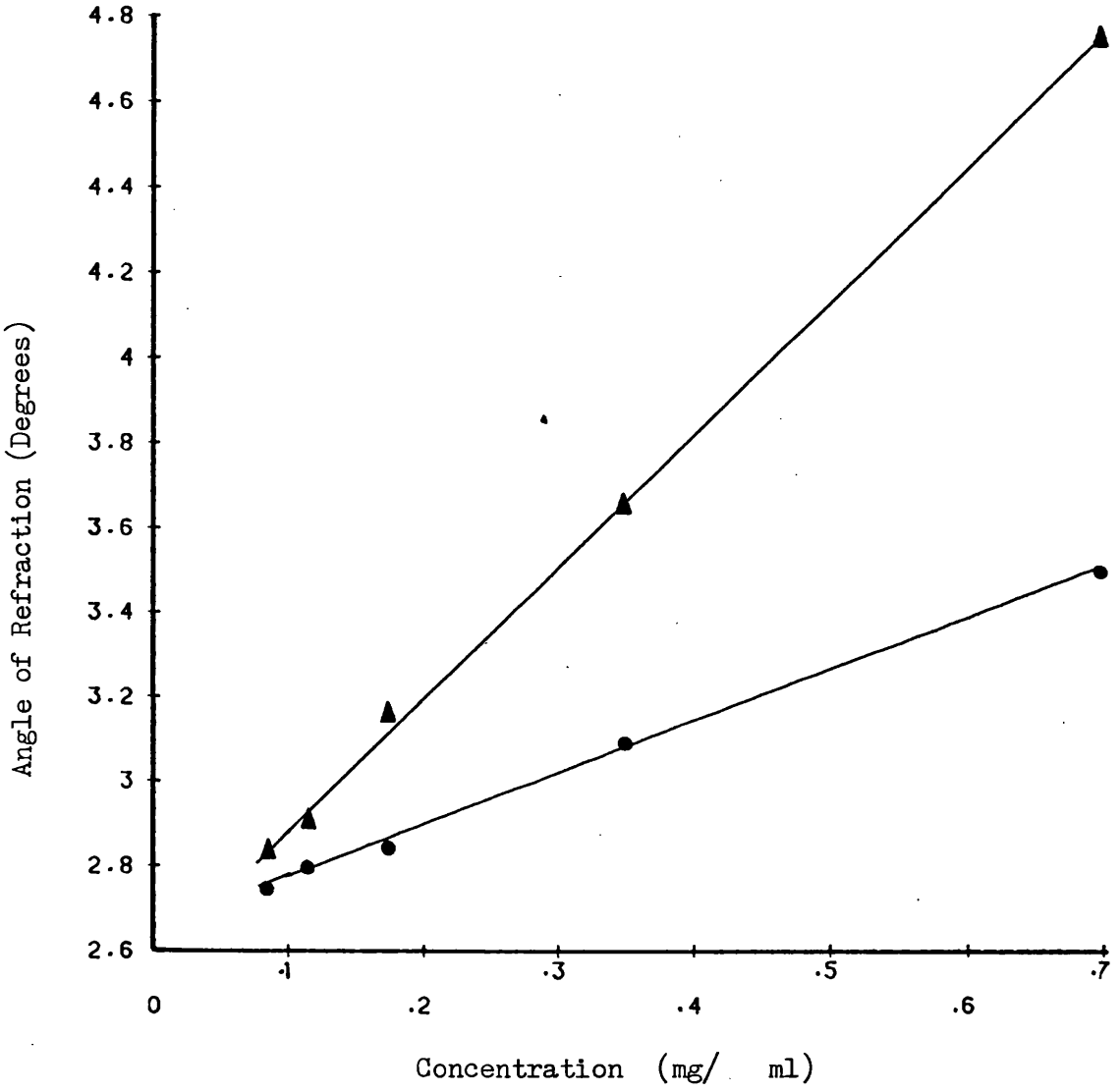


Fig. 3.33 Refractivity of Dextran T70 and Dextran-ATP

(Measurements were taken using an Abbe refractometer at 50 °C using a monochromatic sodium D-line light source)

▲——▲ Dextran-ATP

●——● Dextran T70

refraction could still offer a method of calculating complex concentration with dextran-glycine as a standard. Fig. 3.34 shows curves obtained over the range 0 - 100 mg/ml. These results were reproducible and showed that at concentrations of less than 50 mg/ml the dextran-glycine was a suitable standard for determination of complex concentration.

Estimation of Bound Cofactor

The amount of cofactor in a unit weight of complex was determined by two methods. The first and most simple method was to calculate the number of moles present from the molar extinction coeffient at 254 nm. It was hoped to use this for rapid accurate assessments of subsequent batches. To ensure that this approach was valid, the results were compared with measurements of the total phosphorus present by the methods of Bartlett (1959), to ensure that no hydrolysis of the terminal phosphate group had occurred. Samples of both ATP-dextran and ADP-dextran were measured, see Table 3.35.

Enzyme Availability of Bound Cofactor

The two methods of cofactor determination give good agreement allowing the total bound cofactor to be accurately stated. To assess the availability of the bound cofactor, the complex was assayed using the hexokinase linked assay. As the stoichiometry of the reaction is 1 mol ATP used per mol of NADH formed, the total ATP used at completion can be calculated from the total optical density change at 340 nm. Table 3.36 shows that even at the highest loading achieved essentially

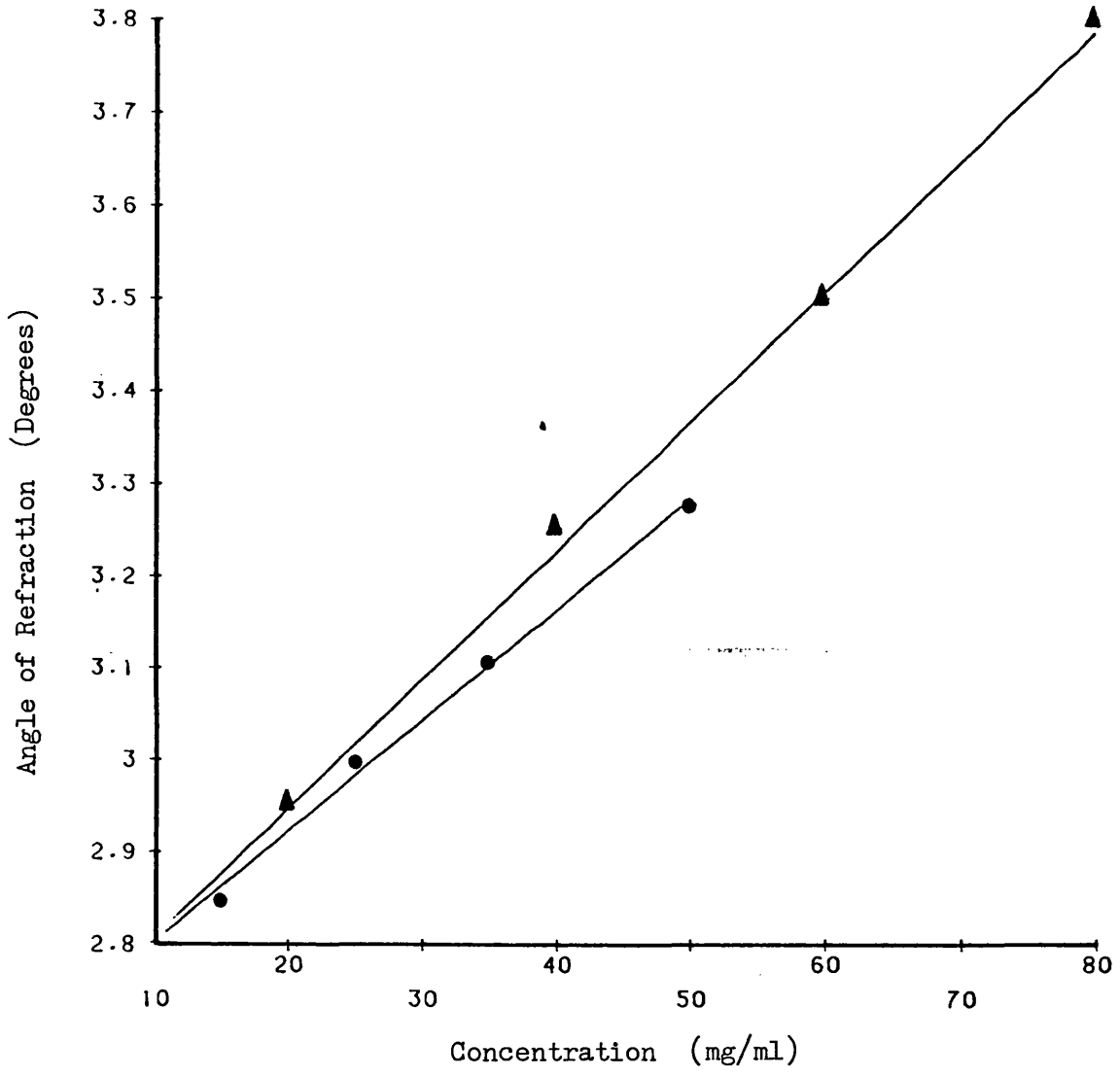


Fig. 3.34 Comparison of Refractivity between Dextran-Glycine and Dextran-ATP

▲——▲ Dextran-Glycine

●——● Dextran-ATP

Table 3.35 Comparison of Cofactor Quantities as Determined by Total Phosphate and E_{254}

Cofactor Complex	Sample Size	Total Phosphate (μmols)	Nucleotide by Phosphate (mM)	Nucleotide by E_{254} (mM)
Dextran-ADP (1)	0.1 ml	0.015	0.075	0.077
Dextran-ADP (2)	0.05 ml	0.33	3.3	3.36
Dextran-ATP (1)	0.4 ml	0.39	0.325	0.310
Dextran-ATP (2)	0.005 ml	0.506	33.8	31.3

Table 3.36 Effect of Degree of Substitution on Cofactor Availability

Number of Groups per Complex molecule	(ATP) mM E_{254}	(ATP) mM Enzymic	% Difference
18	2.41	2.51	4
23	2.55	2.40	6.25
25	2.33	2.43	4.1
26	2.40	2.35	2.1
28	2.40	2.48	3.3
34	2.51	2.40	6.6

all of the cofactor is available to the enzyme.

Enzyme Activities of Dextran-Cofactor Complexes

The demonstration of analytical methods suitable for characterising the complexes produced allowed the effect of incubation time on cofactor binding to be studied. Fig. 3.37 shows the effect of incubation time on the number of molecules of cofactor per molecule of complex. The availability of these groups to enzyme action must be assessed. From a consideration of reactor characteristics the amount of dextran used should be minimised suggesting a highly substituted complex. However, in the more highly substituted complexes the proximity of groups may limit their availability for enzyme attachment.

A range of complexes with differing ATP loadings was produced and the effect of loading on the kinetic constants of hexokinase was studied, see Fig. 3.38.

The complex was tested with a range of enzymes and was shown to be active in each case, emphasising its suitability for use in an enzyme reactor, see Table 3.39.

Stability

The freeze dried activated dextran preparation stored at 0 ^{o}C, showed no reduction in free epoxide groups, as determined by the thiosulphate method, over a period of 6 months.

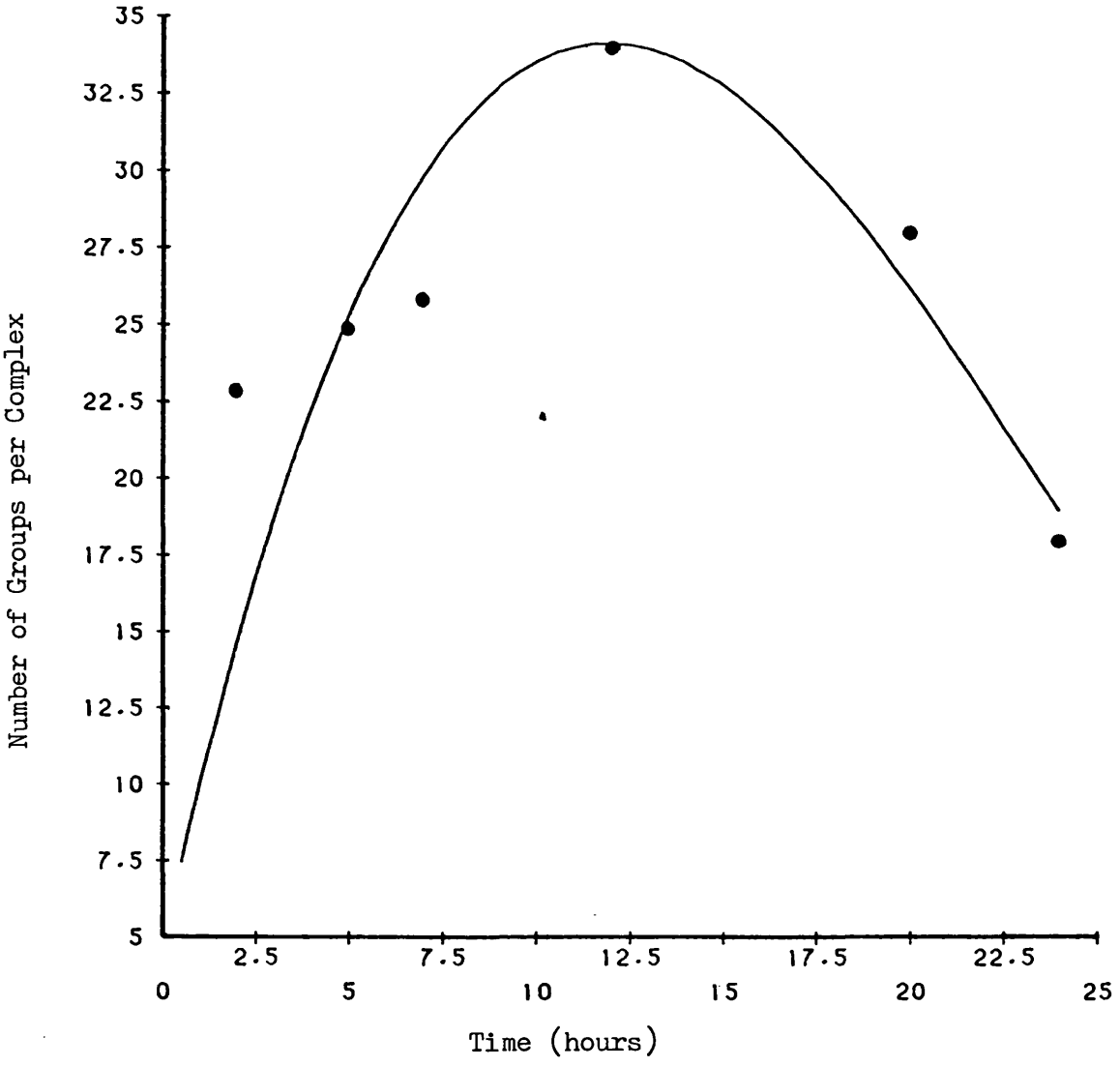


Fig. 3.37 Effect of Incubation Time on ATP Binding to Activated Dextran T40

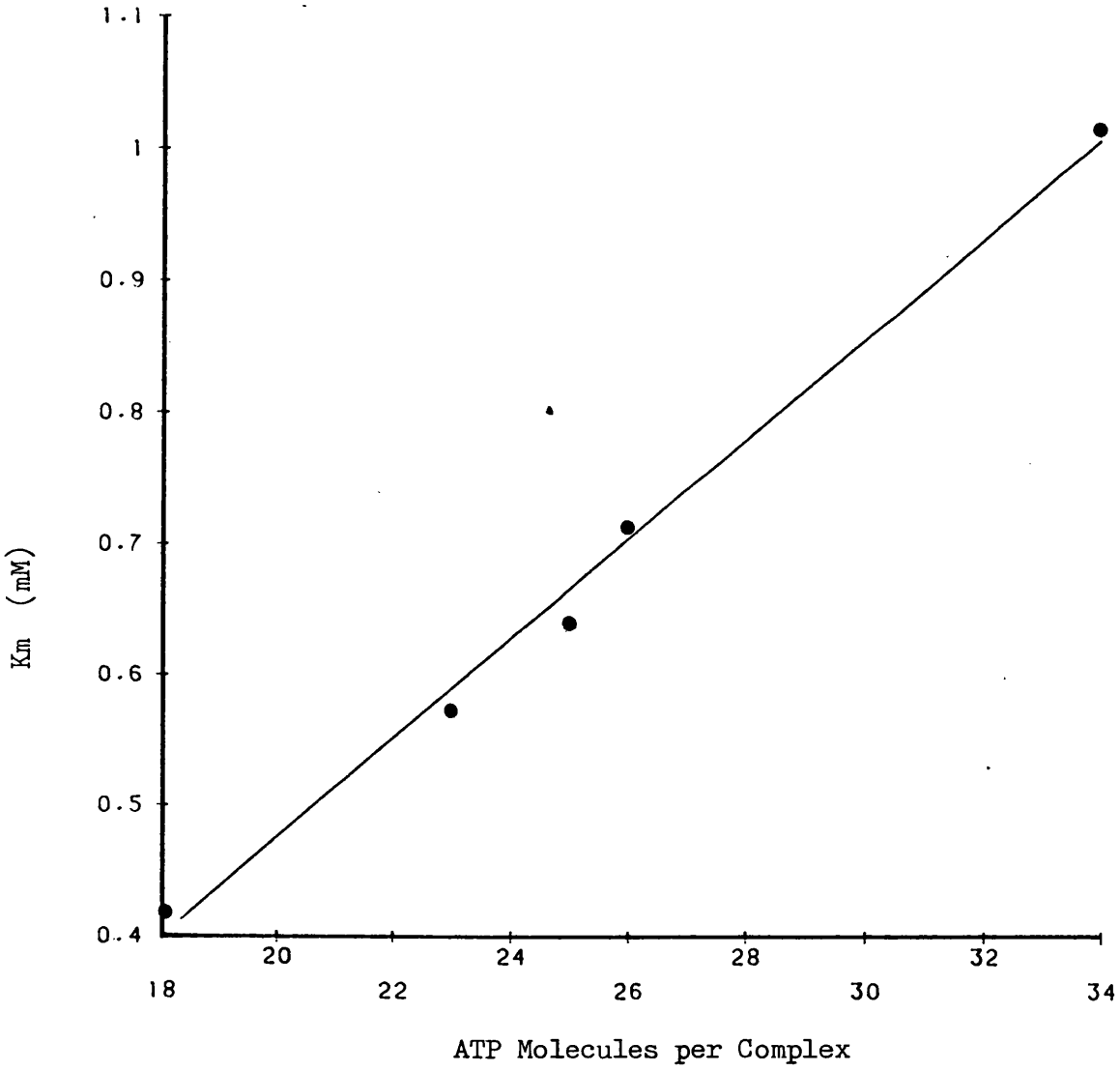


Fig. 3.38a Effect of Cofactor Loading on Apparent Km for Hexokinase

(Kinetic parameters were determined from initial rate studies using the direct linear plot program written by A. Cornish-Bowden. (Henderson, 1978))

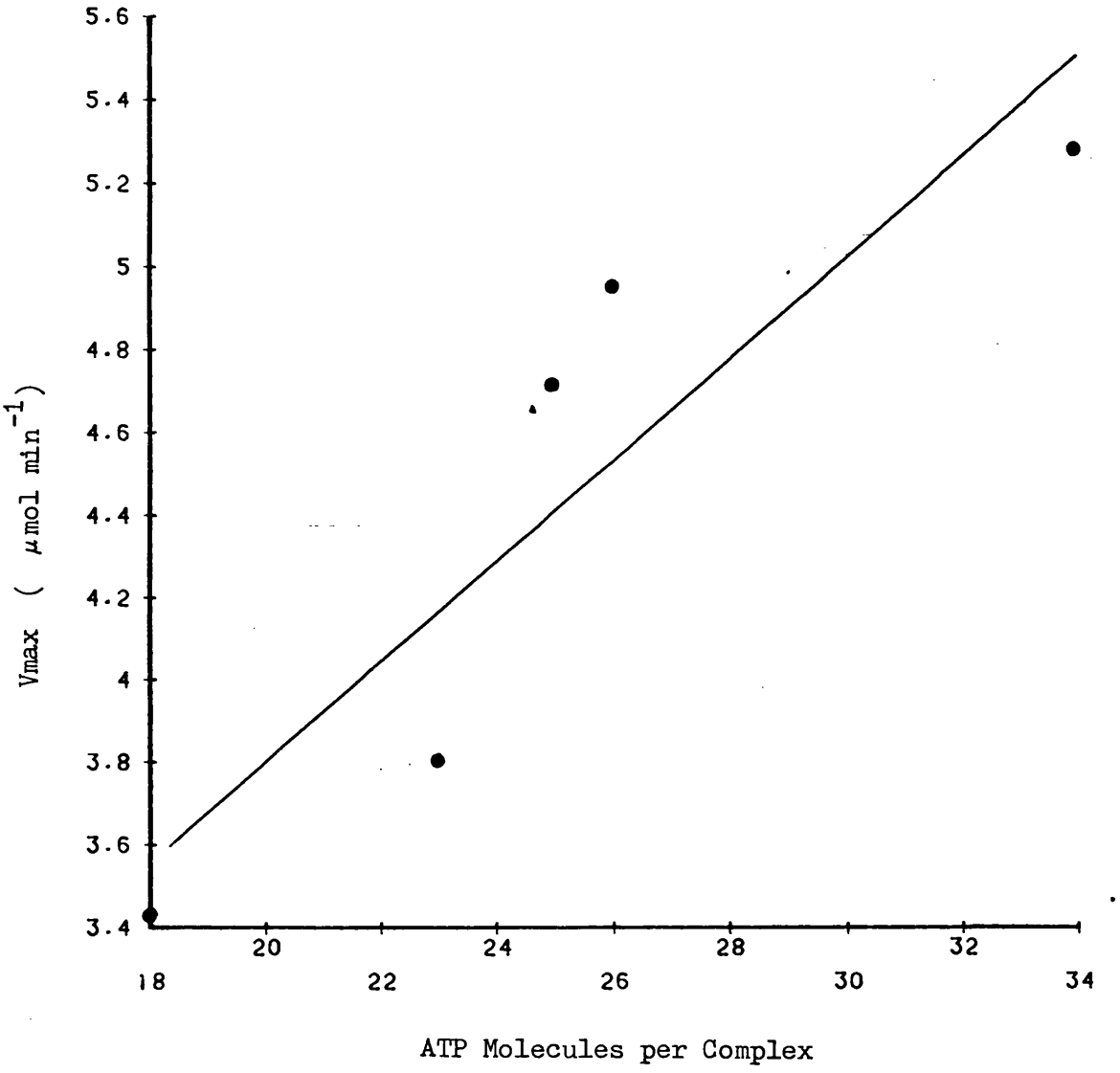


Fig. 3.38b Effect of Cofactor Loading on Vmax with Hexokinase

Table 3.39 Enzyme Activities

Enzyme	Active	Km^{app*} (mM)	$Vmax^{*}$ (μmol/min)
Hexokinase	Yes	0.41	0.44
Acetate Kinase	Yes	0.6	0.31
Myokinase	Yes	-	-
Pyruvate Kinase	Yes	-	-

* Km^{app} and Vmax values quoted represent "typical" values obtained at a cofactor loading of approximately 10 groups/complex.

The immobilized cofactor preparations were found to lose less than 5% enzyme available groups over a period of 3 months at 4 °C. This was the case for both freeze dried samples and those in 25 mM tris buffer, pH 7.

Reactor Studies

Reactor runs were carried out to demonstrate the feasibility of continuously recycling the complex. The initial experiment was designed to show conclusively that recycling was taking place.

The reactor was loaded with unpurified dextran-ATP and was eluted with molar salt until the E_{254} of the effluent returned to zero. Subsequently the reactor was washed through with seven residence volumes of the starting buffer, (25 mM phosphate, 150 mM D-glucose, 50 mM Mg^{2+}, pH 7.5). The run was started with 0.35 mg of hexokinase and allowed to proceed for 4 residence times. At this point the buffer was made 30 mM with respect to acetyl phosphate and 0.06 mg of acetate kinase was injected into the reactor. The run proceeded for another 3 residence times before termination. Aliquots of 1 ml were collected throughout and were assayed for glucose 6-phosphate, see Fig. 3.40.

Having demonstrated cofactor recycling in the reactor, the effect of acetyl phosphate on glucose 6-phosphate production was studied.

The reactor was loaded with 25 μmols purified $ATP^{complex}$, 0.14 mg hexokinase, and 0.06 mg of acetyl kinase, the buffer used was 25 mM phosphate, 50 mM Mg^{2+}, 150 mM D-glucose. Acetyl phosphate concentration was varied throughout the run. Aliquots corresponding to 1 residence

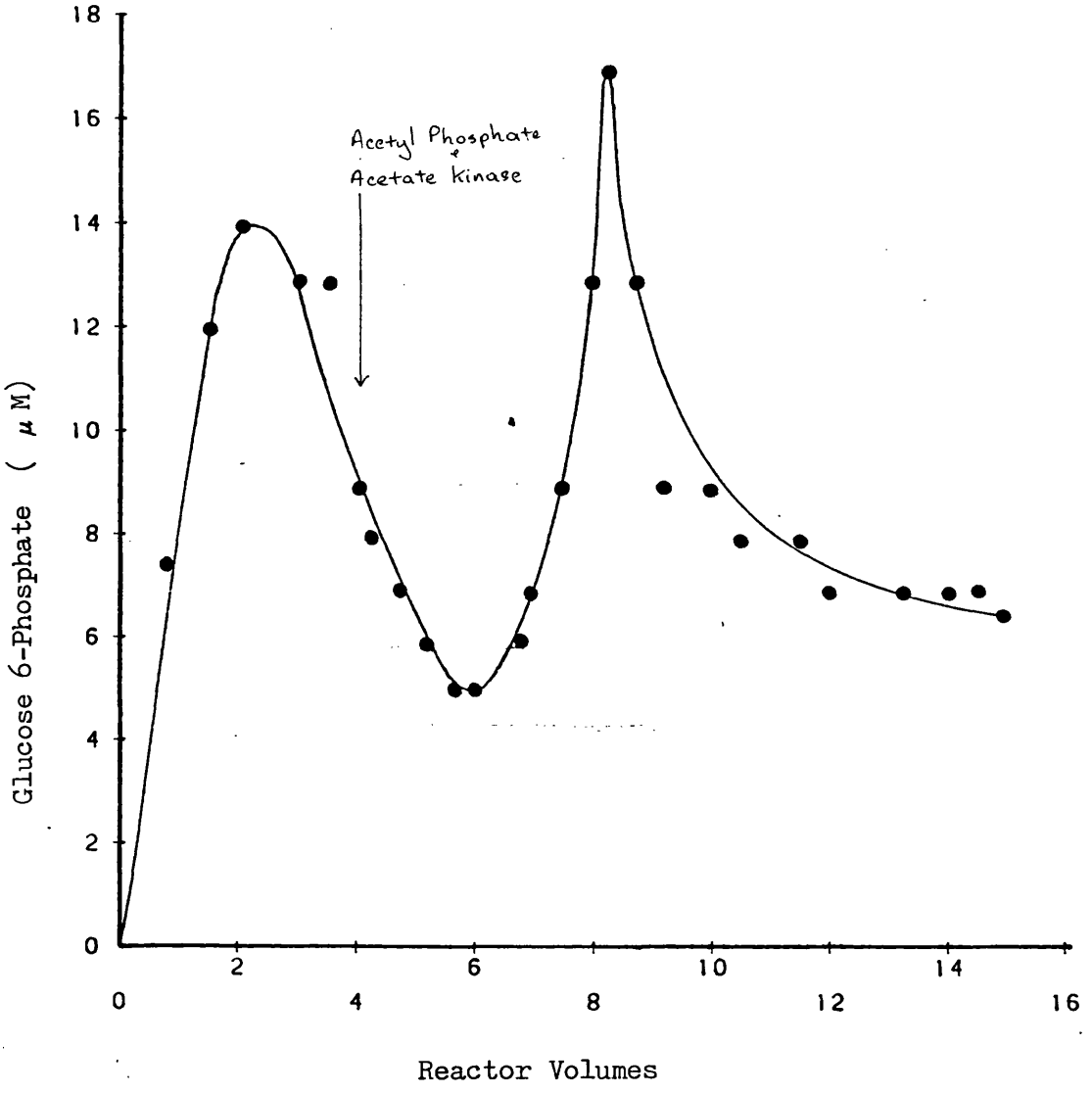


Fig. 3.40 Reactor Run 1

(see text for details)

volume were taken throughout the run, see Fig. 3.41.

Finally an attempt was made to study the effect the acetate kinase/ hexokinase ratio had on the performance of the reactor. The buffer used was as before with an acetyl phosphate concentration of 150 mM. The reactor was 4 mM with respect to immobilized ATP and the reaction was started by the addition of 0.06 mg acetate kinase and 0.14 mg hexokinase. Further additions of acetate kinase was made at 24, 36, 48 and 60 hours, the increment being 0.03 mg in each case. Aliquots comprising 1 residence volume were assayed, see Fig. 3.42.

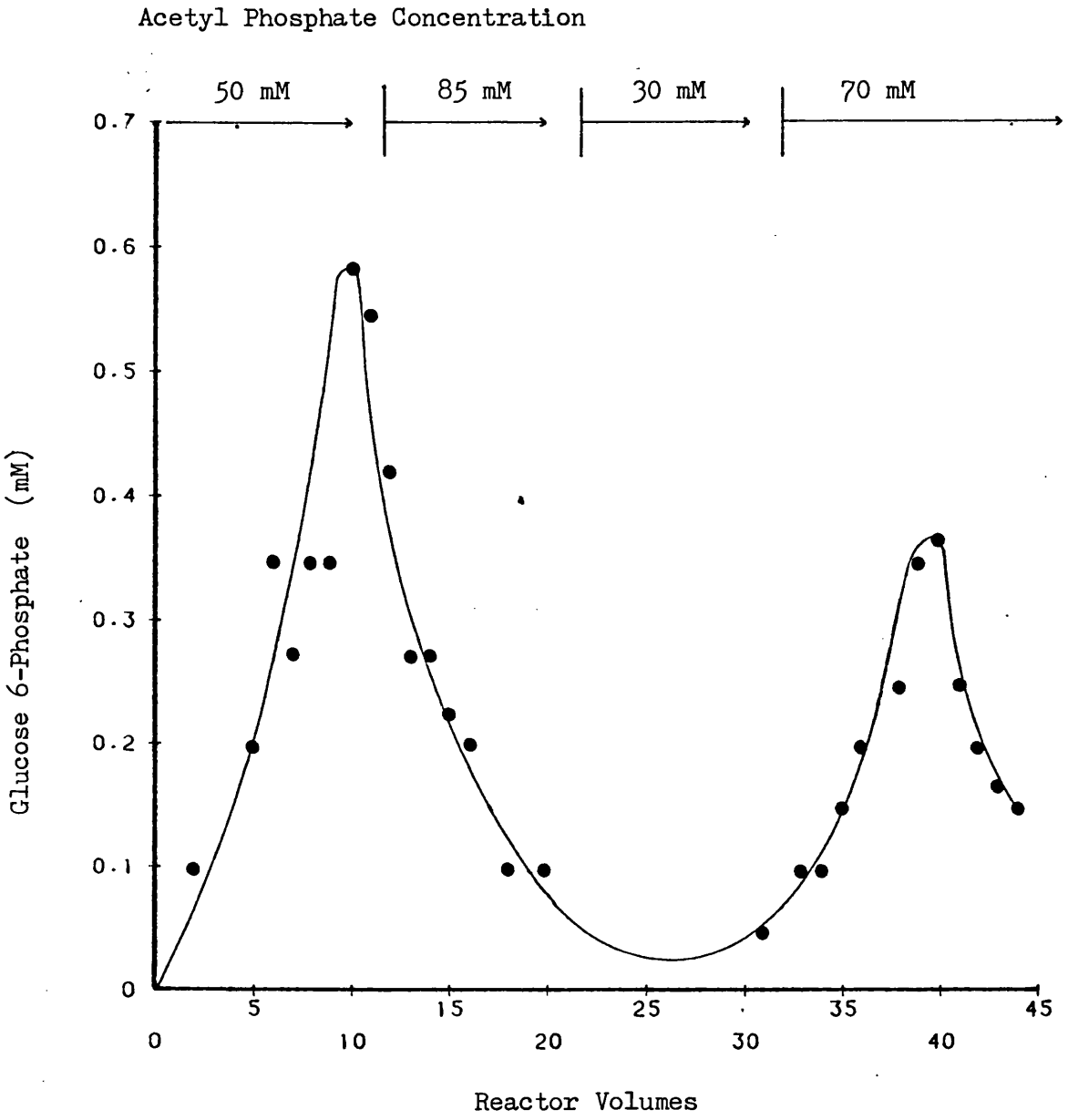


Fig. 3.41 Reactor Run 2

(see text for details)

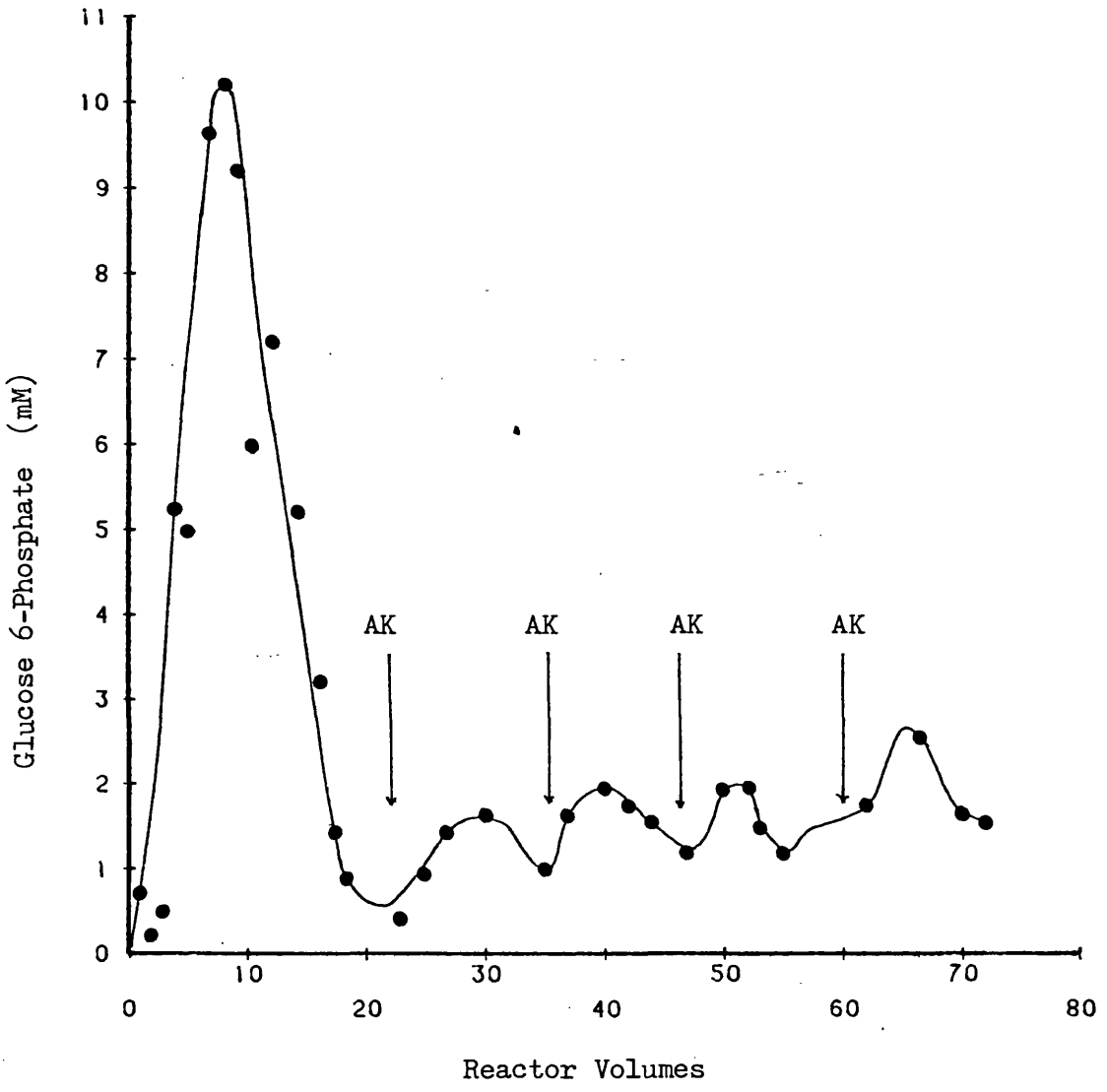


Fig. 3.42 Reactor Run 3

(see text for details)

DISCUSSION

ENZYME THERMISTOR

The enzyme thermistor system used in this study demonstrated the potential of a simplified system. Technical difficulties were encountered in accurately positioning the thermistor in the column material. Positioning was found to be critical with respect to the immobilized enzyme. The use of a resistor to calibrate the system introduced several problems. The bulk of the component caused disruption of the packing and its insulation caused a slow release of the heat generated thus reducing the response. The results show an interesting relationship between flow rate and sensitivity. There would appear to be two factors which could account for this. At higher flow rates, the extent of the reaction would be less as the residence time of substrate in the column would be reduced. However, at higher flow rates the reduced residence time would lessen the effect of energy transfer from the liquid to the column packing material. This would minimise the base line drift in continuous operation. At low flow rates problems of noise were encountered as the reference effect of the differential circuit was lost.

The response of the circuit was shown to tail off at higher energy inputs. This was shown to be accounted for by an increased time to reach steady state. The time taken to reach steady state could be dramatically reduced using higher flow rates but only at the expense of sensitivity. The results suggest that the ideal mode of operation is to use an active enzyme preparation with a reasonably high flow rate. However, care must be taken not to attempt to measure over too

wide a range of concentrations. If a narrow range of concentration is envisaged it should be possible to adjust the conditions to give a linear output.

The results obtained with urease demonstrated the use of the system for measurement of discrete samples. The curve shows typical saturation kinetics and suggests that measurement should be kept to concentrations below the Km value of the substrate if a linear response is required.

The results obtained show a potential of three samples per hour (Fig. 3.8). This figure must be improved if the system is to be widely used. The parameters constraining the sampling time are, flow rate, enzyme activity and detector sensitivity. The degree of interdependence of these parameters prevents their consideration in isolation and so a generalised mode of operation is proposed. Both the flow rate and excitation voltage should be maximised within the practical limitations. The high flow rate should minimise the problems caused by the increased power dissapation of the thermistors. The increased circuit sensitivity should permit the sample size to be decreased, as the residence time is reduced by this approach the enzyme concentration in the column should be maximised. The sensitivity to background noise at higher excitation voltages would be countered by the improved performance of the reference system at higher flow rates.

The use of an adsorption matrix was not successful, as the energy changes were masked by changes resulting from glucose adsorbing onto the column. It was found to be possible to adsorp and desorp enzyme

in situ, suggesting the use of ion exchange resin may be worth investigating in this context.

The adsorption, desorption changes offer a potentially interesting system for assessing potential adsorption systems. It could be used for determining the rate of loading for a column in various buffers.

REACTOR CHARACTERISTICS

The attainment of a steady state pressure in the reactor was shown to be dependent on the ionic strength of the buffer used. The time taken to reach steady state appeared independent of protein concentration.

The use of the dynamic model proposed in Chapter 3 allowed the effects of various parameters on the time taken to reach steady state to be studied. This model predicted percentage of protein at the membrane, not necessarily directly related to transmembrane pressure drop. The results show that the time to steady state is independent of protein concentration but is related to the ratio of flux to back diffusion. The lower the ratio the longer the system takes to reach equilibrium. The effect of buffer strength on transmembrane pressure drop suggests the protein is being salted in. This is described by White, Handler and Smith (1973) as small ions of neutral salts interacting with the ionic groups of protein molecules, diminishing protein molecule interactions and therefore increasing solubility. Although the solubility of albumin in water is not greatly affected, this may well account for a reduction of adsorption effect between soluble albumin and the polarised gel layer.

The effects of pH on solubility shows proteins to be least soluble at their isoelectric point, a factor which has been recognised in the case of ultrafiltration (Melling and Westmacott, 1972). To minimise the effects of concentration polarisation the reactor should be operated where possible at a pH removed from the isoelectric point.

The effects of flux and reactor protein concentration on transmembrane pressure drop was studied as a measure of concentration polarisation. The results showed the exponential relationship predicted by the theory but pressure was not linear with respect to protein concentration at the membrane surface. The results suggested that pressure was a complex function of membrane protein concentration. The concept of incipient gel formation has been proposed to account for concentration polarisation (Kozinski and Lightfoot, 1972).

The results obtained here can be explained in terms of an assumed gel layer, the height and concentration of which is to vary between certain limits. This is in constrast with the originally proposed model where the gel concentration was assumed to be constant. The results of Nakao et al., (1979) and the observation of Porter (1972) throw doubts on this original assumption showing gel concentration to vary with flux and bulk concentration. In an article on pressure effects on enzymes Morild (1981) describes three protein volumes namely, compositional volume, conformational volume and solvation volume. The results suggest that under certain conditions the protein changes from the solvated to the non solvated state, with an accompanied decrease in volume. The extent to which this change occurred would be dictated by factors such as pH, salt concentration, temperature and pressure. The height of the resultant gel layer would be a function of the concentration and the total protein in the gel state. It is unlikely that further compression occurs as Greco et al., (1979) have demonstrated the activity of polarised enzymes. This change in state would obviously not be predicted by the original model as it is a function of external parameters. If the gel concentration could

be accurately predicted, the height of the gel layer could be calculated from the Hagen-Poiselle equation (Nakao et al., 1979) allowing the amount of protein in the gelled state to be calculated. An accurate assessment of the degree of polarisation is essential if the reactor kinetics are to be studied.

The effect of mass transfer on transmembrane pressure drop showed that at low protein loading (less than 0.04% w/v) the effect of volumetric recycle rate was small over the range attainable in the reactor used. Increasing the protein concentration allowed the trend to be followed but did not allow conclusions to be drawn as the range was not great.

The results concur with those of Gacesa (1977) who found that increasing the pulse rate of the recycle pumps had a much greater effect on concentration polarisation than increases in volumetric recycle.

The effect of temperature on concentration polarisation was more dramatic with an observed decrease of 22% in transmembrane pressure drop over the range of 21 - 35 ^{o}C. This result emphasises the danger of studies carried out in isolation. Any assessment of temperature effect on enzyme kinetic constants in the reactor must be compensated for the changes in available enzyme.

The experiments on the effects of pH and ionic strength on concentration polarisation showed that once the gel layer is formed it is essentially unaffected by changes in ionic strength and pH. The initial conditions in eachcase were likely to cause the maximum concentration polarisation thus the subsequent changes do not reduce the gel layer. These results

coupled with the steady state studies show that gel formation is sensitive to pH and ionic strength but that once formed the gel is unaffected by changes in these paramters.

Experiments with the protease Subtilisin carlsberg demonstrated the feasibility of in situ membrane regeneration. The results showed that the most effective method was to minimise the back diffusion to maximise the polarisation on to the membrane. This effectively localises the enzyme in the region of highest substrate concentration. The small molecular weight products are removed through the membrane maximising the rate of protein removal. The problem of removing the protease before reuse of the reactor remains to be solved. Precipitation with trichloroacetic acid is effective but causes problems of concentration polarisation with precipitated protein being deposited on the membrane. The use of autolytic protease preparations may provide the answer by minimising the protein left in the reactor. Certainly the in situ specific hydrolysis of protein offers advantages in cofactor requiring systems if the need to reload with expensive cofactor complex can be avoided.

COFACTOR IMMOBILIZATION

The initial aim of this work was to produce a soluble dextran-ATP complex using the bis oxirane 1-4-butane diol diglycidyl ether. The use of diepoxide for the preparation of affinity ligands (Mansson et al., 1976) and also to form soluble complexes (Coughlin et al., 1976. Fuller and Bright, 1977) has been reported. The method adopted here was to couple adenine nucleotides to dextran T40 using a method based on that proposed by Sundberg and Porath (1974).

The initial experiments showed that reaction conditions could be adjusted to give an activated dextran complex. The critical factor was dextran concentration which was optimised to prevent the formation of an insoluble precipitate. Sundberg and Porath showed that the activation reaction follows a time course of 15 hours; after this point the number of active groups was found to decrease. The experiment with dextran required a method of separating the activated dextran from unactivated epoxide. Precipitation of the complex with ethanol followed by redissolving in distilled water was considered to be the most suitable method. The activated dextran is less easily precipitated than the native form. However, this problem can be overcome by the use of cold ethanol (approximately -20 ^{o}C) and recoveries in excess of 75% can be achieved.

Estimation of the number of epoxide groups on the activated dextran was shown to be extremely pH dependent. Results were found to be unreliable if fluctuations in pH occurred and so all estimations were carried out in a pH stat system using a proportional control system.

The study of the time course of the activation showed the reaction to occur in a time scale of 45 minutes with further incubation leading to a reduction in the number of groups. Some difficulty in redissolving the free dextran complex was found in this experiment. It was noticed that at time points in excess of 1 hour the solubility became markedly reduced.

This suggests that in addition to hydrolysis of groups there is some degree of internal crosslinking occurring. This experiment was repeated with the reaction being stopped by reducing the pH to 5.5 prior to precipitation. The time course of the reaction was the same order, however, the number of active groups was higher. It was also observed that centrifugation of the precipitate accentuated the problem of crosslinking and was avoided in later studies.

The conditions used for binding cofactor to the activated dextran were essentially similar to the method of Sundberg and Porath (1974). Some problems were encountered in the separation of free from bound nucleotide. Column chromotography was discarded as the elution patterns were found to be anomalous. The most probable explanation of these findings is an electrostatic or aromatic interaction with the gel matrix. Purines have been previously reported to show a delayed elution on Sephadex G-10 (Williams, 1972). Similar reactions are believed to cause absorption to polyacrylamide gels. These interactions could not be swamped by high salt concentration and meant that columns would need to be unacceptably long.

Attempts to use a Concanavalin A column also had to be discarded as

although dextrans could be bound, the activated complex was not retained. The degree of substitution appears to prevent binding.

Dialysis, although time consuming was found to be the most convenient method of purification. Samples were dialysed against a 200 volume excess of distilled water with four changes over a period of 48 hours. The amount of free cofactor was determined by elution through a PM 10 membrane and was found to be less than 1% after this time.

The use of ultrafiltration membranes was demonstrated using a stirred ultrafiltration cell and this method was used for some subsequent reactor runs, demonstrating that pre-purification is not essential.

Having established the suitability of dialysis for removal of unbound cofactors, the effect of the time course on the number of bound molecules could be studied. The binding showed a maximum value after 12 hours with a subsequent reduction occurring . The reason for this reduction is not obvious and was not further investigated. The number of cofactor groups were estimated from the published molar extinction coefficients at 254 nm. These values were compared with the measurement of total phosphorus. The values gave good agreement when corrcted for the number of moles of phosphate per mole of cofactor. Thus the molar E_{254} is not appreciably changed when the molecule is coupled to dextran in this manner. Before the kinetic constants of various enzymes for the complex were measured, the percentage of bound cofactor available for enzyme attachment was determined. This was found to be 100% for both acetate kinase and hexokinase at the highest cofactor loading obtained.

The convenience of this method for producing dextran-cofactor complexes at a range of cofactor loadings allowed a study of the effect of this parameter on the kinetic constants obtained with hexokinase. The results show both Km and Vmax to increase with the degree of loading, (Fig.3.38).

Literature reports on the effects of immobilization on apparent Km are conflicting. Fuller and Bright (1977) report a decrease in Km while other workers (Yamazaki et al., 1977. Mosbach, 1978) report increases compared with free cofactor. Increases in Km can be attributed to a range of factors including steric hindrance (Schmidt and Grenner, 1976) and a change in chemical environment (Lowe, 1981).

The increase of Km with loading suggests that steric hindrance is an important factor. As all groups have been shown to be potentially available the results suggest that not all cofactor molecules can be bound simultaneously. Thus although the potential cofactor concentration is equal in each case the concentration available at any given time is reduced at higher loadings. The increase in Vmax with increased loading may be explained in terms of the dextran concnetration in the assay. To achieve equal cofactor concentrations in the assay irrespective of loading requires considerable variation in the dextran concentration. Mosbach (1978) has commented on the effect of large coenzyme matrices on gross diffusional mobility. The increased viscosity leads to a decreased reaction rate and would present severe problems in an ultrafiltration reaction.

Activity of the dextran complex was demonstrated with four enzymes

with the most significant being acetate and adenylate kinase. In view of the conclusions of Langer (1976) it is important that any derivatised cofactor should show activity with acetate kinase as this offers the most promising approach to recycle at present. The use of adenylate kinase may be necessary in some situations to maintain an equilibrium between the three adenine phosphates.

Physical characteristics of the complex were limited to a superficial assessment of molecular weight. Again column chromotography proved unsuitable. The aromatic nature of the procion dye used to 'colour' the dextran samples may well have interacted with the Sepharose column matrix.

Viscosity measurement was the most successful method of molecular weight determination. The experimental results gave a figure of approximately 32,000 daltons for dextran T40. This is in accordance with the quoted weight average and number average values for dextran T40. The value obtained for dextran-cofactor complex shows a weight of approximately 80,000 daltons. The sample used in the determination was prepared using dextran activated for 12 hours and had a measured cofactor concentration of approximately 7 mols/mol original dextran. Fig. 3.29 shows a maximum activation of approximately 200 groups per mole. After 12 hours the remaining groups could be assumed to number approximately 20, to which 7 molecules of ATP were bound. The remainder were blocked with glycine. Thus the molecular weight of the complex can be estimated from the stoichiometry of the reaction :-

1	molecule dextran	40000
7	molecules ATP	3290
200	molecules spacer	41400
10	molecules glycine	750
TOTAL		≃ 85440

Measured value ≃ 80000

These figures give a good agreement with the measured value and suggest that the total complex weight can be approximated if the coupling conditions are known.

Attempts to measure concentrations of dextran-cofactor complexes using a chemical assay for dextran proved unsuccessful. This was attributed to the masking of reducing groups by the activation procedure, however, it was found possible to measure concentration as a function of refractive index if a suitable defined standard was prepared.

Experiments using the immobilized cofactor in the reactor demonstrated the feasibility of in situ purification. In addition it was found that the dual enzyme reaction could recycle the cofactor for in excess of 60 hours. The reaction was shown to be sensitive to acetyl phosphate levels and problems of preventing acetyl phosphate decomposition were encountered. To minimise this problem stock solutions in buffer were made up in small batches and stored on ice prior to being pumped to the reactor. Results suggest the linking enzyme (acetate kinase) is still rate limiting at an activity ratio of 2:1. The initial high conversion corresponds to the utilisation of the ATP present in the reactor (Fig.3.42), after this point the reaction appears limited by

the concentration of acetate kinase.

CONCLUSIONS AND SUGGESTIONS

The problem of cofactor recycle in batch systems has been defined. Shih and Whitesides (1977) demonstrated the production of ATP from adenosine using a three-step reaction. The product was obtained in gram quantities at high percentage conversion. The enzymes used were entrapped in polyacrylamide beads and were subsequently recovered with minimal loss of activity. There have been several investigations into the kinetic parameters covering this type of system (Nemet et al., 1978. Whitesides et al., 1974). In systems of two or more enzymes the problem of optimisation becomes more acute as the inhibition/ activation pattern of magnesium cofactor complexes varies from enzyme to enzyme.

Work on continuous regeneration of cofactors has been less well studied and has been restricted to demonstrating feasibility. Most continuous systems have used membrane ultrafiltration reactors with free enzymes and soluble cofactor analogues (Marshall, 1973. Yamazaki et al., 1977).

Developing cofactor regeneration from a batch to a continuous process requires the solution of three major problems :-

1 Following the reaction

2 Assessing and optimising reactor performance

3 Developing suitable cofactor analogues

Continuous Monitoring

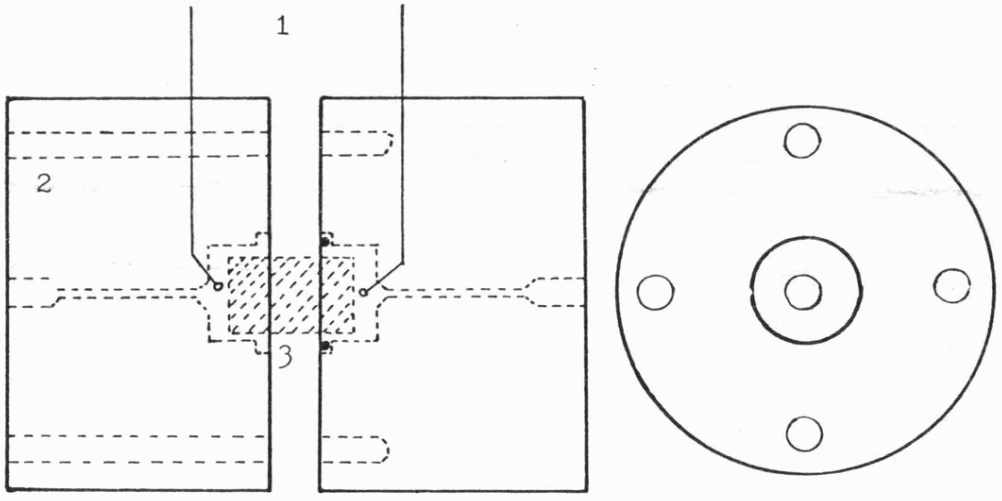
Although other enzyme sensors have been developed, the "flow through" nature of the enzyme thermistor makes it the most suited to on line

measurements of specific biological substrates. The attempt to make a simple enzyme thermistor system based on a differential circuit was only partially successful. The differential circuit used showed promise providing the residence time between the sensors is small. The major limitation of the enzyme thermistor unit was its physical dimensions due to the distance of 4 cm between the thermistors. The use of glass wool supports with a particulate column packing imposes severe restrictions on the range of flow rates attainable. The low flow rates imposed by the construction limit the response time of the unit and increase interference arising from power dissapation at high excitation rates.

In view of these observations the use of ~~immobilized enzyme~~ cartridges is proposed. Enzymes immobilized to a macroporous plug e.g. cellulose matrix, would have the advantage of allowing high flow rates coupled with ease of assembly. The use of a mechanically stable plug would obviate the need for a support column. Thermistors should be mounted proud from the wall of the housing to increase sensitivity, see Fig. 5.1. All connections should be integral with the unit to remove the possibility of short circuits. Construction should be such that cartridges could be replaced without dismantling electrical or fluid connections.

An additional advantage of enzyme cartridges is the potential for reproducibility; mass produced cartridges could be accurately characterised in terms of average life at a variety of conditions. The problem of non linearity may well limit the scope of enzyme detectors for some on line control applications but the potential for discrete sample analysis is extensive.

Fig. 5.1 Proposed Enzyme Thermistor Design



1 Electrical connections

2 Perspex housing

3 Immobilized enzyme cartridge

The circuit used would be identical to Fig. 2.3

Reactor Assessment

In accordance with Marshall (1973) ultrafiltration reactors were considered the most promising system for use with cofactors. Unfortunately, any assessment of reactor performance is complicated by the phenomenon of concentration polarisation. Gacesa (1977) found that in excess of 40% of an enzyme loading could be polarised on the membrane. This figure was found to be in good agreement with the percentage reduction of enzyme activity over the same period.

The gel polarisation model has been widely used to describe concentration polarisation in constant pressure ultrafiltration cells. The model for a constant flux system can be derived in the same manner but a membrane concentration term is used to replace a gel concentration term. In classical systems the gel concentration is assumed to be constant and is calculated by plotting flux against log. bulk concentration and extrapolating to the concentration at zero flux. This assumption has been invalidated by the observations of earlier workers that gel concentrations varied with reactor conditions (Porter, 1972) and by direct measurement (Nakao, 1979). The use of the modified model equation 3.4 (Reactor Studies) shows that membrane concnetration is not linearly related to transmembrane pressure drop. The results suggest that the resistance of the gel to flow is a function of gel concentration and thickness of the gel layer. The thickness of the gel layer can linearly increase but the concentration approaches an assymptotic maximum value. The complexity of this relationship prevents a direct calculation of the percentage of polarised enzymes using the conventional pressure drop correlations for packed beds. The transition of protein

from soluble to gelled form can be explained by the suggestion of Morild (1981) i.e. volume differences between solvated and unsolvated molecules. The results suggest that this transition is a function of a range of parameters including flux, in the case of ultrafiltration reactors.

The most convenient method of determining the protein concentration in the reactor at steady state is by direct measurement or calculation. The results of the dynamic simulation (Appendix 1) show that the steady state concentration is a function of flux and mass transfer, as is the time taken to reach steady state. Flux is accurately preset but the mass transfer coefficient cannot be measured for the experimental system used and the application of the conventional correlations is precluded by the use of pulsed recycle. The problem can be solved indirectly if the bulk concentration is continuously monitored, the time taken to reach steady state being used to estimate the mass transfer coefficient using the dynamic simulation. It would also be of interest to compare the time taken for bulk concentration to reach steady state with the attainment of steady state pressure. This may give some insight to the transition of protein from soluble to gelled state.

The effect of other parameters on concentration polarisation were much as would be expected. The recycle rates attainable were all in the laminar flow region and gave only marginal reductions in transmembrane pressure drop. Temperature gave a more dramatic effect, but in practice the choice of temperature may well be dictated by alternative factors. Salt concentration and pH were shown to have little effect once a gel layer had been formed; however, salt concentration should be optimised

to maximise the solubility of the proteins used in the reactor. The reactions envisaged will probably dictate the pH but where possible the isoelectric point of the protein should be avoided.

The use of proteases showed that in situ membrane regeneration was possible and that the rate of reaction could be increased by polarising the proteases on to the membrane. The problem of removing traces of protease from the reactor must be considered. Chemical precipitation was found to be unsuitable but autolysing proteases may be useful.

Cofactor Analogues

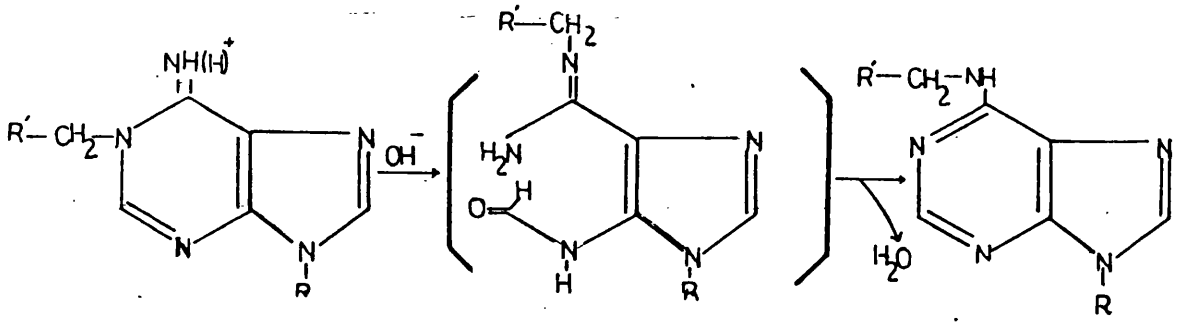
The bis epoxide coupling of ATP and ADP to dextran proved to be a straightforward technique. The time taken to reach maximum activation of the dextran was 45 minutes, compared with a time of 12 hours with Sepharose 6B (Sundberg and Porath, 1974). The preparation was optimised to the point where a freeze dried activated dextran complex could be produced which was totally stable for six months at 0 ^{o}C. Maximum coupling of ATP was obtained after 12 hours. This reaction could be carried out in an ultrafiltration reactor and the excess cofactor removed prior to commencing the run. Alternatively, the sample could be dialysed against distilled water, again the complex was stable for at least three months.

Even at the maximum cofactor loading of 34 molecules per complex, all the cofactor was available for enzyme attachment. The complex was used with a range of enzymes and was demonstrated to be active with all tried.

The effect of degree of substitution on the kinetic constants of hexokinase was studied. The Km and Vmax both increased if the rate was plotted against the concentration of cofactor. However, if the concentration of complex was used, the Km showed little variation. Fig. 5.2 shows a plot of 'complex' Km against degree of substitution. This result suggests that with the highly substituted gels all the cofactor moieties are equally available to the enzyme. The increase in Vmax and elevated Km with the complex at increasing substitution suggest that substitution should be maximised thus decreasing the amount of complex in the reactor and minimising any concentration polarisation problems.

The position of coupling to the cofactor was not extensively investigated. Previous reports have shown alkylating reagents like epoxides react at the nucleophilic N^1 nitrogen of the purine base. The resultant N^1 alkyl derivative undergoes the characteristic Dimroth rearrangement (Fig. 5.3) when exposed to hydroxide ions.

Fig. 5.3



The rate of this rearrangement has been found to be first order with

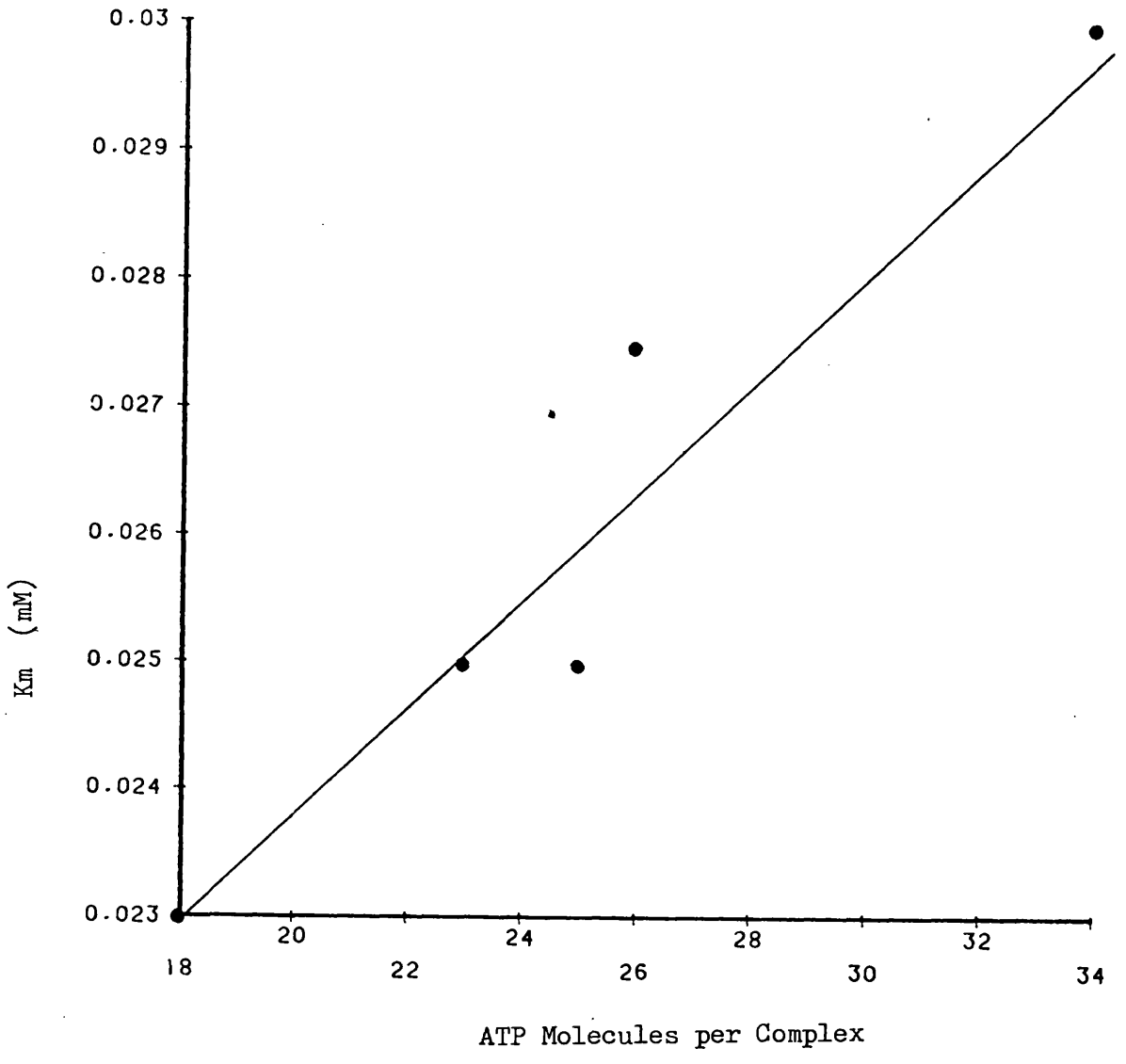


Fig. 5.2 Variation of 'Complex' Km^{app} with Cofactor Loading

respect to the hydroxide ion concentration (Macon and Wolfenden, 1966).

The evidence for the rearrangement has come from several workers. Fuller and Bright (1977) demonstrated a characteristic spectral shift from λmax 260 to 267. Zappelli et al., (1977) and Le Goffic et al., (1980) confirmed C^6 amino alkyl derivatives using H^1 NMR spectroscopy. However, in the case of the $NADP^+$ derivative prepared by Zappelli et al., there was evidence for some side chain modification of the ribose region.

The half life studies of Marcqn and Wolfenden (1966) suggest that not all of the N^1 form would be converted during the reaction time used here. Thus the complex produced might represent a heterogeneous mixture of two or more types of attachment. This was suggested by the qualitative demonstration of the coupling of inosine diphosphate. Thus the measured Km for ATP may represent a composite value incorporating the 'true' Km value for each individual species.

For example, for two substrates present in constant proportion the Km^{app} would be :-

$$Km^{app} = \frac{1 + R}{\frac{1}{K'} + \frac{R}{K}}$$

where :-

K, K'	Km's of the two substrates
R	ratio of the concentration of the two substrates

Attempts were made to measure the quantity of vicinal OH groups of the cofactor ribose moieties, after modification with bis oxirane. The method involved periodate oxidation with subsequent titration of the iodate released. These attempts were unsuccessful due to interactions of the titrated sodium thiosulphate with unblocked epoxide groups, thus preventing any assessment of the possibility of coupling through the ribose hydroxyls. The method of Satake et al., (1960) for assaying free amino groups may allow some quantitative estimations of binding position to be made.

The reactor studies were designed to demonstrate the feasibility of the continuous system. Problems of continuously providing a labile substrate to the reactor will need to be investigated further, and in the case of acetyl phosphate a concentrated feedstock may need to be used to allow the solution to be kept at low temperature prior to pumping to the reactor. The optimisation of the ratio of enzymes in the reactor must be considered, however, this must be carried out individually for every system envisaged.

The work of Nemet et al., (1978) in the regeneration of native cofactors shows that the differing inhibition/activation characteristics of enzymes require that reactor conditions be a compromise. The use of myokinase should be avoided where possible as the equilibrium set up between the three phosphorylated states increases the difficulty in predicting ideal conditions. The next step in a study of continuous cofactor regeneration must be its demonstration for complex synthesis. The most likely process at present is the enzymic synthesis of the antibiotic 'gramicidin S'. Although not of commercial interest it

would serve to demonstrate the feasibility of a multi substrate system requiring ATP regeneration (Nemet et al., 1978. Vandamme, 1981).

At this point the reactor design should be changed from a plate and frame to a hollow fibre system. This would remove the size limitations of the system used here and allow comparison with existing industrial reactors utilising simple reactions. In addition detailed cost assessments as described by Flaschel and Wandrey (1979) would be facilitated.

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APPENDIX 1

Listing of C.S.S.L. Program "Polarisation"

(based on equations 3.7-10. (Reactor Studies))

```
  1     program Polarisation
  2     initial
  3     constant k=1.6e-04, j=8.76e-03, y1=6.25e-09, y2=0.0, ...
  4     ya=6.25e-09,yb=0.0, tfin=600.0
  5     end initial
  6     dynamic
  7     derivative ferm
  8     cinterval ci=10.0
  9     v1=j*y1
 10     v2=k*y2
 11     f1=v2-v1
 12     f2=v1-v2
 13     y1=integ(f1,ya)
 14     y2=integ(f2,yb)
 15     end derivative
 16     termt(t.gt.tfin)
 17     end dynamic
 18     terminal
 19     end terminal
 20     end program
```

Continuous System Simulation Language (version 4)

Supported by Simulation Services, Chatsworth, Ca.

TABLE OF VARIABLES VARIABLE NAMES AND UNITS

FOR PROGRAM POLARISATION.

VARIABLE NAME	VARIABLE	UNITS	VALUE
j	Flux	cm/s	8.76e-03
k	Mass transfer	cm/s	1.6e-04
ya (y1)	Cb	mole/cm3	6.25e-09
yb (y2)	Cm	mole/cm3	0.0
ci	Interval	s	10.0
tfin	Integ. period	s	600.0